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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/55, 9/16, 1/19, 1/20, 1/21, A01H 5/00, A23K 1/00, 1/165, C12Q 1/44, 1/68, C12N 15/82 // (C12N 1/19, C12R 1:84) (C12N 1/21, C12R 1:01, 1:125, 1:19)		A3	(11) International Publication Number: WO 97/48812 (43) International Publication Date: 24 December 1997 (24.12.97)
(21) International Application Number: PCT/CA97/00414 (22) International Filing Date: 13 June 1997 (13.06.97) (30) Priority Data: 60/019,735 14 June 1996 (14.06.96) US 08/744,779 6 November 1996 (06.11.96) US 08/862,531 23 May 1997 (23.05.97) US (71) Applicant (for all designated States except US): HER MAJESTY THE QUEEN IN RIGHT OF CANADA, represented by THE DEPARTMENT OF AGRICULTURE AND AGRI-FOOD CANADA [CA/CA]; Lethbridge Research Station, P.O. Box 3000, Main Lethbridge, Alberta T1J 4B1 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): CHENG, Kuo, Joan [CA/CA]; 2015 - 6th Avenue South, Lethbridge, Alberta T1J 1C2 (CA). SELINGER, Leonard, Brent [CA/CA]; 114 Laval Court West, Lethbridge, Alberta T1K 4G3 (CA). YANKE, Lindsey, Jay [CA/CA]; 1520 - 23rd Avenue N. #18, Lethbridge, Alberta T1H 4X9 (CA). BAE, Hee, Dong [KR/KR]; Eung-Sup Shim, 114-5, Chi-dong, Paldal-gu,		Suwon, Seoul 442-060 (KR). ZHOU, Luming [CA/US]; 1227 East Brickyard Road #203, Salt Lake City, UT 84106 (US). FORSBERG, Cecil, Wallace [CA/CA]; 44 Hands Drive, Guelph, Ontario N1G 3H3 (CA). (74) Agent: McKAY-CAREY, Mary, Jane; McKay-Carey & Com- pany, 10155 - 102nd Street, 2125 Commerce Place, Edmon- ton, Alberta T5J 4G8 (CA). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW. ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW). Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. (88) Date of publication of the international search report: 26 March 1998 (26.03.98)	
(54) Title: DNA SEQUENCES ENCODING PHYTASES OF RUMINAL MICROORGANISMS			
(57) Abstract Phytases derived from ruminal microorganisms are provided. The phytases are capable of catalyzing the release of inorganic phosphorus from phytic acid. Preferred sources of phytases include <i>Selenomonas</i> , <i>Prevotella</i> , <i>Treponema</i> and <i>Megasphaera</i> . A purified and isolated DNA encoding a phytase of <i>Selenomonas ruminantium</i> JY35 (ATCC 55785) is provided. Recombinant expression vectors containing DNAs encoding the phytases and host cells transformed with DNAs encoding the phytases are also provided. The phytases are useful in a wide range of applications involving the dephosphorylation of phytate, including, among other things, use in animal feed supplements.			

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EE	Estonia						

INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/CA 97/00414

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/55 C12N9/16 C12N1/19 C12N1/20 C12N1/21
 A01H5/00 A23K1/00 A23K1/165 C12Q1/44 C12Q1/68
 C12N15/82 //(C12N1/19,C12R1:84),(C12N1/21,C12R1:01,1:125,1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H A23K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	<p>EP 0 420 358 A (GIST BROCADES NV) 3 April 1991 see page 2, line 10-12</p> <p>see page 4, line 47-55 see page 7; table 1 see page 11, line 30-50; example 2 see page 19; example 9 see page 28; claims 22,23,28-31 --- -/-</p>	<p>30</p> <p>1,2,11, 12,14, 15,18, 21,22, 24,36, 39-42, 48,51, 52,58, 69,71, 72,74</p>



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

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E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

22 January 1998

Date of mailing of the international search report

03.02.98

Name and mailing address of the ISA

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Macchia, G

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 97/00414

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	EP 0 449 375 A (GIST BROCADES NV ;MOGEN INT (NL)) 2 October 1991 see page 2, line 11-13 see page 3, line 45-54 see page 7, line 28-55 see page 12, line 40-58; example 10 ---	30 1,27,36, 51,52, 55,58, 69,71, 72,74
X A	WO 94 03072 A (PANLABS INC ;NEVALAINEN HELENA K M (AU); ALKO LTD (FI); PALOHEIMO) 17 February 1994 see page 1, paragraph 2 see page 2, paragraph 1 see page 28, paragraph 1-2 see page 53, paragraph 6 ---	30 43,45, 69,71, 72,74
X A A	PUNJ M.L. ET AL.: "Utilization of phytin phosphorus by rumen microorganisms" THE INDIAN VETERINARY JOURNAL, vol. 46, no. 10, 1969, pages 881-886, XP002044300 see page 885 --- WO 93 16175 A (GIST BROCADES NV) 19 August 1993 see page 5, line 16 - page 6, line 7 -----	30 12,13 69,71, 72,74

INTERNATIONAL SEARCH REPORT

Int. application No.
PCT/CA 97/00414

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/CA 97/00414

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-67 75-77

Isolated nucleic acid encoding a phytase of a ruminal microorganism, as in Seq.ID:1. Expression construct comprising it, transformed host cells and transgenic plants thereof. *Selenomonas ruminantium* JY35 (ATCC 55785). Phytase of a ruminal microorganism as in Seq.ID:2. Feed composition comprising feedstuff treated with said phytase, feed additive comprising microorganisms expressing said phytase. Method for producing said phytase. Method for producing a transgenic plant expressing said phytase. Method for improving dietary phytate utilization by an animal, via use of said phytase. Method to identify a nucleic acid encoding a phytase by means of at least part of Seq.ID:1.

2. Claims: 68-74

A method for assaying phytase activity of a microorganism.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 97/00414

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0420358 A	03-04-91	AU 636673 B	06-05-93
		AU 6501190 A	28-04-91
		BG 60108 A	15-10-93
		CA 2042054 A	28-03-91
		CN 1051058 A	01-05-91
		DE 420358 T	12-10-95
		EP 0779037 A	18-06-97
		ES 2072834 T	01-08-95
		JP 4506007 T	22-10-92
		LT 1527 A,B	26-06-95
		LV 10310 A,B	20-10-94
		WO 9105053 A	18-04-91
		PL 167790 B	30-11-95
		PL 168470 B	29-02-96
		US 5436156 A	25-07-95
EP 0449375 A	02-10-91	AU 649447 B	26-05-94
		AU 7765691 A	21-10-91
		AU 632941 B	14-01-93
		AU 7776691 A	21-10-91
		CA 2056396 A	24-09-91
		EP 0449376 A	02-10-91
		IL 97645 A	18-03-97
		JP 6501838 T	03-03-94
		JP 6502296 T	17-03-94
		WO 9114782 A	03-10-91
		WO 9114772 A	03-10-91
		US 5543576 A	06-08-96
WO 9403072 A	17-02-94	US 5593963 A	14-01-97
		AU 4787993 A	03-03-94
		CA 2141431 A	17-02-94
		EP 0655890 A	07-06-95
		FI 950409 A	30-03-95
WO 9316175 A	19-08-93	JP 9505201 T	27-05-97
		AU 3628493 A	03-09-93
		EP 0626010 A	30-11-94
		FI 943707 A	10-08-94



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ :</p> <p>C12N 15/55, 9/16, 1/19, 1/20, 1/21, A01H 5/00, A23K 1/00, 1/165, C12Q 1/44, 1/68, C12N 15/82 // (C12N 1/19, C12R 1:84) (C12N 1/21, C12R 1:01, 1:125, 1:19)</p>	A2	<p>(11) International Publication Number: WO 97/48812</p> <p>(43) International Publication Date: 24 December 1997 (24.12.97)</p>									
<p>(21) International Application Number: PCT/CA97/00414</p> <p>(22) International Filing Date: 13 June 1997 (13.06.97)</p> <p>(30) Priority Data:</p> <table border="0"> <tr> <td>60/019,735</td> <td>14 June 1996 (14.06.96)</td> <td>US</td> </tr> <tr> <td>08/744,779</td> <td>6 November 1996 (06.11.96)</td> <td>US</td> </tr> <tr> <td>08/862,531</td> <td>23 May 1997 (23.05.97)</td> <td>US</td> </tr> </table> <p>(71) Applicant (for all designated States except US): HER MAJESTY THE QUEEN IN RIGHT OF CANADA, represented by THE DEPARTMENT OF AGRICULTURE AND AGRI-FOOD CANADA [CA/CA]; Lethbridge Research Station, P.O. Box 3000, Main Lethbridge, Alberta T1J 4B1 (CA).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): CHENG, Kuo, Joan [CA/CA]; 2015 - 6th Avenue South, Lethbridge, Alberta T1J 1C2 (CA). SELINGER, Leonard, Brent [CA/CA]; 114 Laval Court West, Lethbridge, Alberta T1K 4G3 (CA). YANKE, Lindsey, Jay [CA/CA]; 1520 - 23rd Avenue N. #18, Lethbridge, Alberta T1H 4X9 (CA). BAE, Hee, Dong [KR/KR]; Eung-Sup Shim, 114-5, Chi-dong, Paldal-gu,</p>		60/019,735	14 June 1996 (14.06.96)	US	08/744,779	6 November 1996 (06.11.96)	US	08/862,531	23 May 1997 (23.05.97)	US	<p>Suwon, Seoul 442-060 (KR). ZHOU, Luming [CA/US]; 1227 East Brickyard Road #203, Salt Lake City, UT 84106 (US). FORSBERG, Cecil, Wallace [CA/CA]; 44 Hands Drive, Guelph, Ontario N1G 3H3 (CA).</p> <p>(74) Agent: McKAY-CAREY, Mary, Jane; McKay-Carey & Company, 10155 - 102nd Street, 2125 Commerce Place, Edmonton, Alberta T5J 4G8 (CA).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>
60/019,735	14 June 1996 (14.06.96)	US									
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<p>(54) Title: DNA SEQUENCES ENCODING PHYTASES OF RUMINAL MICROORGANISMS</p> <p>(57) Abstract</p> <p>Phytases derived from ruminal microorganisms are provided. The phytases are capable of catalyzing the release of inorganic phosphorus from phytic acid. Preferred sources of phytases include <i>Selenomonas</i>, <i>Prevotella</i>, <i>Treponema</i> and <i>Megasphaera</i>. A purified and isolated DNA encoding a phytase of <i>Selenomonas ruminantium</i> JY35 (ATCC 55785) is provided. Recombinant expression vectors containing DNAs encoding the phytases and host cells transformed with DNAs encoding the phytases are also provided. The phytases are useful in a wide range of applications involving the dephosphorylation of phytate, including, among other things, use in animal feed supplements.</p>											

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DNA SEQUENCES ENCODING PHYTASES OF RUMINAL MICROORGANISMS

Field of the Invention

This invention relates to phytases derived from ruminal microorganisms.

Background of the Invention

Although the plant constituents of livestock feedstuffs are rich in phosphorus, inorganic phosphorus supplementation is required to obtain good growth performance of monogastric animals. Phytic acid (*myo*-inositol hexaphosphoric acid) generally occurs as a complex of calcium, magnesium and potassium salts and/or proteins, and is the predominant form of phosphorus in cereals, oil seeds, and legumes, and accounts for 1 to 3% of the seed dry weight and 60 to 90% of the total phosphorus present in seeds (Graf, 1986). However, monogastric animals (e.g., swine, poultry and fish) utilize phytate poorly or not at all because they are deficient in gastrointestinal tract enzymes capable of hydrolyzing phytate. Phytate passes largely intact through the upper gastrointestinal tract, where it may decrease the bioavailability of nutrients by chelating minerals (e.g., calcium and zinc), binding amino acids and proteins (Graf, 1986) and inhibiting enzymes. Phytate phosphorus in manure poses a serious pollution problem, contributing to eutrophication of surface waters in areas of the world where monogastric livestock production is intensive.

Production inefficiencies and phosphorus pollution caused by phytate may be effectively addressed by phytase supplementation of diets for monogastric animals. Phytases catalyze the hydrolysis of phytate to *myo*-inositol and inorganic phosphate, which are then absorbed in the small intestine. In addition to decreasing phosphorus supplementation requirements and reducing the amount of phytate pollutants released, phytases also diminish the antinutritional effects of phytate.

Phytases are produced in animal and plant (predominantly seeds) tissues and by a variety of microorganisms (U.S. Patent No. 3,297,548; Shieh and Ware, 1968; Ware and Shieh, 1967). Despite the array of potential phytase sources, only soil fungi (*Aspergillus niger* or *Aspergillus ficuum*) are currently used for commercial production of phytase. The phytase produced by *A. ficuum* possesses greater specific activity (100 units/mg of protein (wherein units are defined as μ moles of

1 phosphate released per minute)) and thermostability compared to those phytases
2 that have been characterized from other microorganisms (European Patent
3 Application No. 0,420,358 (van Gorcum *et al.*, 1991) and U.S. Patent No. 5,436,156
4 (van Gorcum *et al.*, issued July 25, 1995)). The *A. ficuum* phytase is an acid phytase
5 and exhibits little activity above pH 5.5 (Howson and Davis, 1983; van Gorcum *et al.*,
6 1991). Consequently, activity is limited to a relatively small region of the monogastric
7 digestive tract, in which the pH ranges from 2-3 (in the stomach) to 4-7 (in the small
8 intestine).

9 Although the idea of phytase supplementation of monogastric diets was
10 proposed more than 25 years ago (U.S. Patent No. 3,297,548, Ware and Shieh,
11 1967), the high cost of enzyme production has restricted the use of phytase in the
12 livestock industry. In North America, supplemental phytase is generally more
13 expensive than phosphorus supplements. In some circumstances, the cost of
14 phytase utilization may be partially offset if the use of this enzyme also decreases
15 the need for supplementation of a second nutrient such as calcium. The use of
16 phytase in North America is likely to increase as swine and poultry populations
17 increase and as public pressures force a reduction in pollution associated with
18 livestock production. Higher costs of phosphorus supplements and legislation
19 requiring the use of phytase have made the use of this supplement more common
20 in Europe and parts of the Orient than in North America. Governments of the
21 Netherlands, Germany, Korea and Taiwan have enacted or are enacting legislation
22 to reduce the phosphorus pollution created by monogastric livestock production.

23 A more effective means of increasing phytase utilization is through cost
24 reduction. The cost of phytase can be reduced by decreasing production costs
25 and/or producing an enzyme with superior activity. Recent advances in
26 biotechnology may revolutionize the commercial enzyme industry by offering
27 alternative, cost effective methods of enzyme production. Application of recombinant
28 DNA technology has enabled manufacturers to increase the yields and efficiency of
29 enzyme production, and to create new products. The original source organism need
30 no longer limit the production of commercial enzymes. Genes encoding superior
31 enzymes can be transferred from organisms such as anaerobic bacteria and fungi,
32 typically impractical for commercial production, into well characterized industrial

1 microbial production hosts (e.g., *Aspergillus* and *Bacillus* spp.). As well, these genes
2 may be transferred to novel plant and animal expression systems.

3 Unlike monogastric animals, ruminants (e.g., cattle, sheep) readily utilize the
4 phosphorus in phytic acid. It has been demonstrated that phytases are present in
5 the rumen, and it has been proposed that ruminants reared on high grain diets (rich
6 in phytate) do not require dietary phosphorus supplementation due to these ruminal
7 phytases. A single report has attributed this phytase production to ruminal
8 microorganisms (Raun *et al.*, 1956), but overall, the unique capacity of ruminants to
9 utilize phytate has largely been ignored. Raun *et al.* (1956) prepared microbial
10 suspensions by centrifugal sedimentation (Cheng *et al.*, 1955). Those microbial
11 suspensions were almost certainly contaminated with microscopic particles of plant
12 material. Since plants produce phytases, the study was inconclusive as to whether
13 plant phytases or microbial phytases produced the observed activity. Although Raun
14 *et al.* have raised the possibility that ruminal phytase production may be attributable
15 to ruminal microorganisms, this possibility has not been explored.

16 In view of the foregoing, there remains a need for low cost phytases having
17 biochemical characteristics well suited for use in animal feed supplements.

18 19 Summary of the Invention

20 The inventors have discovered that the rumen is a rich source of
21 microorganisms which produce phytases having biochemical characteristics (such
22 as temperature and pH stability, low metal ion sensitivity and high specific activity)
23 desirable for industrial applications such as animal feed supplementation and inositol
24 production. Ruminal microorganisms tolerate anaerobic conditions and may be
25 either facultative or obligate anaerobes. Ruminal microorganisms may be
26 prokaryotes (i.e. bacteria) or eukaryotes (i.e. fungi, protozoa). As used herein, the
27 term "ruminal microorganisms" includes microorganisms isolated from the digesta or
28 feces of a ruminant animal.

29 Ruminal bacterial species which have been identified as providing particularly
30 active phytases includes *Selenomonas ruminantium*, *Prevotella* sp, *Treponema*
31 *bryantii* and *Megaphaera elsdenii*. *Prevotella* and *Selenomonas* are Gram negative
32 anaerobic rods from the family Bacteroidaceae.

1 In accordance with the present invention, DNA sequences encoding novel and
2 useful phytases derived from ruminal microorganisms are provided.

3 A phytase gene (*phyA*) from *Selenomonas ruminantium* strain JY35 has been
4 cloned and sequenced, and the nucleotide sequence of the *phyA* gene is provided.
5 The invention extends to DNA sequences which encode phytases and which are
6 capable of hybridizing under stringent conditions with the *phyA* gene sequence. As
7 used herein, "capable of hybridizing under stringent conditions" means annealing to
8 a subject nucleotide sequence, or its complementary strand, under standard
9 conditions (ie. high temperature and/or low salt content) which tend to disfavor
10 annealing of unrelated sequences. As used herein, "conditions of low stringency"
11 means hybridization and wash conditions of 40 - 50°C, 6 X SSC and 0.1% SDS
12 (indicating about 50 - 80% homology). As used herein, "conditions of medium
13 stringency" means hybridization and wash conditions of 50 - 65°C, 1 X SSC and
14 0.1% SDS (indicating about 80 - 95% homology). As used herein, "conditions of high
15 stringency" means hybridization and wash conditions of 65 - 68°C, 0.1 X SSC and
16 0.1% SDS (indicating about 95-100% homology).

17 As used herein, the term "phytase" means an enzyme capable of catalyzing
18 the removal of inorganic phosphorus from a *myo*-inositol phosphate.

19 As used herein, the term "*myo*-inositol phosphate" includes, without limitation,
20 *myo*-inositol hexaphosphate, *myo*-inositol pentaphosphate, *myo*-inositol
21 tetraphosphate, *myo*-inositol triphosphate, *myo*-inositol diphosphate and *myo*-inositol
22 monophosphate.

23 As used herein, "phytate" means the salt of *myo*-inositol hexaphosphoric acid.

24 The invention extends to the *S. ruminantium* JY35 (ATCC 55785) organism
25 itself, and to methods for identifying and isolating this and other ruminal
26 microorganisms exhibiting phytase activity as well as methods for isolating, cloning
27 and expressing phytase genes from ruminal microorganisms exhibiting phytase
28 activity using part or all of the *phyA* gene sequence as a probe.

29 The invention further extends to methods for assaying phytase production by
30 a microorganism whereby false positive results caused by microbial acid production
31 are eliminated. Colonies of microorganisms are grown on a growth medium
32 containing phytate. The medium is contacted with an aqueous solution of cobalt

1 chloride and the medium is then examined for zones of clearing. Preferably, rather
2 than examining the medium immediately, the solution of cobalt chloride is removed
3 and the medium is contacted with aqueous solutions of ammonium molybdate and
4 ammonium vanadate and then examined for zones of clearing. False positive results
5 which occur when acid-forming microbes produce zones of clearing are avoided.

6 The invention extends to expression constructs constituting a DNA encoding
7 a phytase of the present invention operably linked to control sequences capable of
8 directing expression of the phytase in a suitable host cell.

9 The invention further extends to host cells which have been transformed with,
10 and express, DNA encoding a phytase of the present invention, and to methods of
11 producing such transformed host cells. As used herein "host cell" includes animal,
12 plant, yeast, fungal, protozoan and prokaryotic host cells.

13 The invention further extends to transgenic plants which have been
14 transformed with a DNA encoding a phytase of the present invention so that the
15 transformed plant is capable of expressing the phytase and to methods of producing
16 such transformed plants. As used herein, "transgenic plant" includes transgenic
17 plants, tissues and cells.

18 Phytases of the present invention are useful in a wide variety of applications
19 involving the dephosphorylation of phytate. Such applications include use in animal
20 feed supplements, feedstuff conditioning, human nutrition, and the production of
21 inositol from phytic acid. Phytases of the present invention may also be used to
22 minimize the adverse effects of phytate metal chelation. The high phytate content
23 of certain feedstuffs such as soy meal decreases their value as protein sources for
24 fish, monogastric animals, young ruminants and infants because the phytate
25 decreases the bioavailability of nutrients by chelating minerals, and binding amino
26 acids and proteins. Treatment of such feedstuffs with the phytases of the present
27 invention will reduce their phytate content by phytase mediated dephosphorylation,
28 rendering the feedstuffs more suitable for use as protein sources. Accordingly, the
29 invention extends to novel feed compositions comprising feedstuffs treated with a
30 phytase of the present invention, and feed additives containing a phytase of the
31 present invention. Such feed compositions and additives may also contain other
32 enzymes, such as, proteases, cellulase, xylanases and acid phosphatases. The

1 phytase may be added directly to an untreated, pelletized, or otherwise processed
2 feedstuff, or it may be provided separately from the feedstuff in, for instance, a
3 mineral block, a pill, a gel formulation, a liquid formulation, or in drinking water. The
4 invention extends to feed inoculant preparations comprising lyophilized
5 microorganisms which express phytases of the present invention under normal
6 growing conditions. With respect to these feed inoculant preparations, "normal
7 growing conditions" mean culture conditions prior to harvesting and lyophilization of
8 the microorganisms. The microorganisms express phytases during growth of the
9 microbial cultures in large-scale fermenters. The activity of phytases in the
10 microorganisms is preserved by lyophilization of the harvested microbial
11 concentrates containing the phytase.

12 The invention further extends to a method for improving an animal's utilization
13 of dietary phosphate by feeding the animal an effective amount of a phytase of the
14 present invention. As used herein "an effective amount" of a phytase means an
15 amount which results in a statistically significant improvement in phosphorus
16 utilization by the animal. Phytate phosphorus utilization may be evidenced by, for
17 instance, improved animal growth and reduced levels of phytate in animal manure.

18 19 **Brief Description of Drawings**

20 Figure 1 is a photograph showing the effect of counterstaining agar medium
21 containing phytate on zones of clearing produced by acid production or phytase
22 activity. Phytate agar was inoculated with *S. bovis* (top of left petri dish) and *S.*
23 *ruminantium* JY35 (bottom of left petri dish) and incubated for 5 d at 37°C. The
24 colonies were scraped off and the medium counterstained with cobalt chloride and
25 ammonium molybdate/ammonium vanadate solutions (right petri plate).

26 Figure 2 is a graph illustrating the growth (protein) and phytase production of
27 *S. ruminantium* JY35 in modified Scott and Dehority (1965) broth.

28 Figure 3A, 3B and C show transmission electron micrographs of cells from a
29 mid-exponential phase culture of *S. ruminantium* JY35 incubated for reaction product
30 deposition by phytase using sodium phytate as the substrate. Untreated control cells
31 are shown for comparison in Figures 3D, 3E and 3F.

1 Figure 4 is a graph illustrating the phytase pH profile for washed *S.*
2 *ruminantium* JY35 cells in five different buffers.

3 Figure 5 is a graph illustrating the pH profile of *S. ruminantium* JY35 MgCl₂ cell
4 extract in five different buffers.

5 Figure 6 is a graph illustrating the temperature profile of *S. ruminantium* JY35
6 MgCl₂ cell extract.

7 Figure 7 is a graph illustrating the effect of ions (10 mM) on *S. ruminantium*
8 JY35 phytase activity (Ctr = control).

9 Figure 8 is a graph illustrating the effect of sodium phytate concentration on
10 *S. ruminantium* JY35 phytase activity.

11 Figure 9 is a zymogram developed for confirmation of phytase activity.
12 Concentrates (10 x) of *S. ruminantium* JY35 MgCl₂ extract (lanes B - E), low
13 molecular weight markers (lane F, BioRad Laboratories Canada Ltd, Mississauga,
14 Ontario) and *A. ficuum* phytase (Sigma, 1.6 U, lane A) were resolved by SDS-PAGE
15 in a 10% polyacrylamide gel. Lanes A to E were stained for phytase activity and
16 Lane F was stained with Coomassie brilliant blue.

17 Figure 10 is a photograph of a phytate hydrolysis plate assay for phytase
18 activities of *E. coli* DH5 α transformed with pSrP.2 (top), pSrP.2 Δ *SphI* (bottom left),
19 and pSrP16 (bottom right). Zones of clearing were visible after incubating the plates
20 at 37°C for 48 h.

21 Figure 11 is a Southern blot analysis using the 2.7-kb fragment from pSrP.2
22 as a probe against *SphI* digested pSrP.2 DNA (lane B) and *HindIII* digested genomic
23 DNA isolated from *S. ruminantium* JY35 (lane C). Digoxigenin labelled *HindIII*
24 digested Lambda DNA was run as a molecular weight standard in lane A.

25 Figure 12 is a physical map of pSrP.2. A 2.7-kb fragment, from a *Sau3A*
26 partial digest of *S. ruminantium* JY35 genomic DNA, was cloned into the *BamHI* site
27 of pUC18. This fragment contains the entire gene encoding the phytase from *S.*
28 *ruminantium* JY35. The location of a *BamHI* site lost as a result of the ligation is
29 indicated in square brackets.

30 Figure 13 is a schematic representation of the deletion analysis of the *S.*
31 *ruminantium* phytase gene. The position of *phyA* is indicated by the horizontal arrow.

1 The hatched boxes indicate segments of the 2.7-kb *Sau3A* fragment carried by
2 different plasmid derivatives. Phytase activity is indicated in the panel to the right.

3 Figure 14 is a zymogram developed for phytase activity. *E. coli* DH5 α
4 (pSrP.2) cells (lane A), *E. coli* DH5 α (pSrP.2 Δ *SphI*) cells (lane B), and low molecular
5 weight markers (lane C, BioRad Laboratories) were resolved by SDS-PAGE in a
6 10% polyacrylamide gel. Lanes A and B were stained for phytase activity and Lane
7 C was stained with Coomassie brilliant blue.

8 Figure 15 is the nucleotide sequence of the *S. ruminantium* JY35 phytase
9 gene (*phyA*) (SEQ ID NO. 1) and its deduced amino acid sequence (SEQ ID NO. 2).
10 Nucleotide 1 corresponds to nt 1232 of the 2.7-kb insert of pSrP.2. The putative
11 ribosome binding site is underlined and shown above the sequence as R.B.S. The
12 signal peptidase cleavage site, predicted by the method of von Heijne (1986) is
13 indicated by the |. The N-terminal amino acid sequence of the phytase secreted by
14 *E. coli* (pSrPf6) is underlined.

15 16 Detailed Description of the Preferred Embodiment

17 The rumen is a complex ecosystem inhabited by more than 300 species of
18 bacteria, fungi and protozoa. Screening these organisms for phytase activity
19 requires the ability to discriminate the phytase activity of individual isolates. This
20 may be accomplished through the assessment of pure cultures from a stock culture
21 collection or separation and cultivation of individual cells through cultural techniques
22 (e.g., streak plate, dilution and micromanipulation). Standard aseptic, anaerobic
23 techniques described for bacteria, fungi and protozoa may be used to accomplish
24 this goal.

25 Suitable enzyme assays are necessary for screening microbial isolates in
26 ruminal fluid samples and from culture collections, and for cloning phytase genes.
27 Assays for measuring phytase activity in solutions have been described in the
28 literature. Sample solutions are typically assayed for phytase activity by measuring
29 the release of inorganic phosphorus (P_i) from phytic acid (Raun *et al.*, 1956; van
30 Hartingsveldt *et al.*, 1993). Phytase activity may also be detected on solid media.
31 Microorganisms expressing phytase produce zones of clearing on agar media
32 containing sodium or calcium phytate (Shieh and Ware, 1968; Howson and Davis,

1 1983). However, the solid media assays described in the literature were found to be
2 unsatisfactory for screening ruminal bacteria for phytase activity because of the false
3 positive reactions of acid-producing bacteria such as *Streptococcus bovis*. To
4 overcome this problem, a two-step counterstaining procedure was developed in
5 which petri dishes containing solid medium are flooded first with an aqueous cobalt
6 chloride solution and second with an aqueous ammonium molybdate/ammonium
7 vanadate solution. Following this treatment only clearing zones produced by enzyme
8 activity are evident (Figure 1).

9 Using the above solutions and solid medium assays, 345 isolates from the
10 Lethbridge Research Centre (Lethbridge, Alberta, Canada) culture collection were
11 screened for phytase activity (Table 1). A total of 29 cultures with substantial
12 phytase activity were identified, including 24 of the genus *Selenomonas* and 5 of the
13 genus *Prevotella*. Twelve of these cultures (11 *Selenomonas* isolates and 1
14 *Prevotella* isolate) had phytase activities substantially higher than the other positive
15 cultures (Table 2).

16 The phytase of *S. ruminantium* JY35 (deposited May 24, 1996 with the
17 American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland,
18 20852-1776, as ATCC 55785) was selected for further examination and compared
19 to a commercial phytase (Gist-brocades nv, Delft, The Netherlands) from *Aspergillus*
20 *ficuum* NRRL 3135 (van Gorcum *et al.*, 1991 and 1995). The phytase of *S.*
21 *ruminantium* JY35 (ATCC 55785) is constitutively expressed, exported from the cell
22 and associated with the cell surface. The pH (Figure 5) and temperature (Figure 6)
23 profiles of the *S. ruminantium* JY35 (ATCC 55785) phytase were comparable, if not
24 more suited to industrial production, than are those of the commercial *A. ficuum*
25 NRRL 3135 phytase. These results demonstrated the potential of ruminal and
26 anaerobic microbes as sources of phytases with characteristics superior to phytases
27 currently being produced by industry.

28 Microbial genes encoding selected enzymes can be cloned by a variety of
29 methods. Gene libraries (genomic DNA and/or cDNA) are constructed by standard
30 methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1990) and screened for the desired
31 gene. The screening methodology may utilize heterologous probes, enzyme activity

1 or results generated during purification of the gene product, such as N-terminal and
2 internal amino acid sequence data and antibodies.

3 Using the solid medium phytase assay developed to detect phytase activity
4 produced by ruminal microbes, a *S. ruminantium* JY35 (ATCC 55785) gene library
5 was screened for positive clones. Of 6000 colonies examined, a single colony was
6 identified as a phytase positive clone by a large zone of clearing around the colony.
7 This clone carried a 5.5-kb plasmid comprising a 2.7-kb *Sau3A* DNA fragment
8 inserted into cloning vector pUC18. The newly isolated 2.7-kb *Sau3A* DNA fragment
9 was used as a probe in Southern blot hybridizations. Under high stringency
10 conditions, a discrete band could be detected for *S. ruminantium* isolate JY35 (ATCC
11 55785), but not for *Prevotella* sp. 46/5², *E. coli* DH5 α or *A. ficuum* NRRL 3135.

12 Plasmid DNA isolated from the newly isolated clone and introduced into *E. coli*
13 cells by transformation produced ampicillin-resistant, phytase-positive CFUs.
14 Zymogram analysis of cell extracts from *E. coli* DH5 α cells carrying the 2.7-kb *Sau3A*
15 DNA fragment from *S. ruminantium* JY35 (ATCC 55785) revealed a single activity
16 band with an estimated molecular mass of 37 kDa. Deletion and DNA sequence
17 analyses were used to identify the gene (*phyA*) which encoded the phytase
18 responsible for the activity observed in recombinant *E. coli* clones. The N-terminal
19 amino acid sequence of the purified 37-kDa phytase expressed in *E. coli* cells
20 carrying *phyA* matched the N-terminal amino acid sequence of the mature phytase
21 predicted from the cloned *phyA* sequence. This indicated conclusively that the
22 nucleotide sequence encoding the phytase had been isolated. The nucleotide
23 sequence and deduced amino acid sequence are shown in Figure 15.

24 As with other genes, it is possible to use the characterized phytase coding
25 sequence in a variety of expression systems for commercial enzyme production.
26 Application of recombinant DNA technology has enabled enzyme manufacturers to
27 increase the volume and efficiency of enzyme production, and to create new
28 products. The original source organism need no longer limit the production of
29 commercial enzymes. Genes encoding superior enzymes can be transferred from
30 organisms such as anaerobic bacteria and fungi, typically impractical for commercial
31 production, into well characterized industrial microbial production hosts (e.g.,

1 *Aspergillus*, *Pichia*, *Trichoderma*, *Bacillus* spp.). As well, these genes may be
2 transferred to novel plant and animal expression systems.

3 Industrial strains of microorganisms (e.g., *Aspergillus niger*, *Aspergillus*
4 *ficuum*, *Aspergillus awamori*, *Aspergillus oryzae*, *Trichoderma reesei*, *Mucor miehei*,
5 *Kluyveromyces lactis*, *Pichia pastoris*, *Saccharomyces cerevisiae*, *Escherichia coli*,
6 *Bacillus subtilis* or *Bacillus licheniformis*) or plant hosts (e.g., canola, soybean, corn,
7 potato) may be used to produce phytase. All systems employ a similar approach to
8 gene expression. An expression construct is assembled to include the protein
9 coding sequence of interest and control sequences such as promoters, enhancers
10 and terminators. Other sequences such as signal sequences and selectable
11 markers may also be included. To achieve extracellular expression of phytase, the
12 expression construct of the present invention utilizes a secretory signal sequence.
13 The signal sequence is not included on the expression construct if cytoplasmic
14 expression is desired. The promoter and signal sequence are functional in the host
15 cell and provide for expression and secretion of the coding sequence product.
16 Transcriptional terminators are included to ensure efficient transcription. Ancillary
17 sequences enhancing expression or protein purification may also be included in the
18 expression construct.

19 The protein coding sequences for phytase activity are obtained from ruminal
20 microbial sources. This DNA may be homologous or heterologous to the expression
21 host. Homologous DNA is herein defined as DNA originating from the same species.
22 For example, *S. ruminantium* may be transformed with DNA from *S. ruminantium* to
23 improve existing properties without introducing properties that did not exist previously
24 in the species. Heterologous DNA is defined as DNA originating from a different
25 species. For example, the *S. ruminantium phyA* may be cloned and expressed in *E.*
26 *coli*.

27 It is well known in the biological arts that certain amino acid substitutions can
28 be made in protein sequences without affecting the function of the protein.
29 Generally, conservative amino acid substitutions are tolerated without affecting
30 protein function. Similar amino acids can be those that are similar in size and/or
31 charge properties, for example, aspartate and glutamate and isoleucine and valine
32 are both pairs of similar amino acids. Similarity between amino acid pairs has been

1 assessed in the art in a number of ways. For example, Dayhoff et al. (1978) in Atlas
2 of Protein Sequence and Structure, Volume 5, Supplement 3, Chapter 22, pages
3 345-352, which is incorporated by reference herein; provides frequency tables for
4 amino acid substitutions which can be employed as a measure of amino acid
5 similarity. Dayoff et al.'s frequency tables are based on comparisons of amino acid
6 sequences for proteins having the same function from a variety of evolutionary
7 different sources.

8 It is also well-known that often less than a full length protein has the function
9 of the complete protein, for example, a truncated protein lacking an N-terminal,
10 internal or a C-terminal protein often ha the biological and/or enzymatic activity of the
11 complete natural protein. Gene truncation experiments involving *phyA* have
12 confirmed that the truncated protein may retain the function of the intact protein.
13 *Exherichia coli* clones expressing PhyA missing N-terminal amino acids 1-37 or 1058
14 (SEQ ID NO. 2) showed phytase positive phenotypes. In contrast, no phytase
15 activity could be detected for a clone expressing PhyA missing acids 307-346 (SEQ
16 ID NO. 2). Those of ordinary skill in the art know how to make truncated protein and
17 proteins with internal deletions. In the present invention, the function of a truncated
18 phytase protein or an internally deleted phytase protein can be readily tested using
19 the assay described hereinbelow and n view of what is generally known in the art.

20 Substituted, internally-deleted and truncated rumina phytase derivatives which
21 retain substantially the same enzymatic activity as a phytase specifically disclosed
22 herein are considered equivalents of the exemplified phytase and are within the
23 scope of the present invention, particularly where the specific activity of the
24 substituted, internally-deleted or truncated phytase derivative is at least about 10%
25 of the specifically exemplified phytase. The skilled artisan can readily measure the
26 activity of a rumina phytase, truncated phytase, internally-deleted phytase or
27 substituted phytase using the assay procedures taught herein and in view of what
28 is generally known in the art.

29 This invention includes structurally variant phytases derived from a phytase
30 of a rumina microorganisms, particularly those derived from a phytase specifically
31 disclosed herein, that are substantially functionally equivalent to that phytase as
32 assayed as described herein in view of what is generally known in the art.

1 Structurally variant, functional equivalents of the phytases of this invention include
2 those phytase of rumina microorganisms having a contiguous amino acid sequence
3 as in the phytase amino acid sequence disclosed herein (SEQ ID NO. 2), particularly
4 those variant phytase which have a contiguous amino acid sequence of a phytase
5 of a rumina microorganism that is a contiguous sequence at least about 25 amino
6 acids in length.

7 The present invention also provides the starting material for the construction
8 of phytases with properties that differ from those of the enzymes isolated herein.
9 The genes can be readily mutated by known procedures (e.g., chemical, site
10 directed, random polymerase chain reaction mutagenesis) thereby creating gene
11 products with altered properties (e.g., temperature or pH optima, specific activity or
12 substrate specificity).

13 Various promoters (transcriptional initiation regulatory region) may be used
14 according to the present invention. The selection of the appropriate promoter is
15 dependent upon the proposed expression host. Choices of promoters may include
16 the promoter associated with the cloned protein coding sequence or promoters from
17 heterologous sources as long as they are functional in the chosen host. Examples
18 of heterologous promoters are the *E. coli tac* and *trc* promoters (Brosius *et al.*, 1985),
19 *Bacillus subtilis sacB* promoter and signal sequence (Wong, 1989), *aox1* and *aox2*
20 from *Pichia pastoris* (Ellis *et al.*, 1985), and oleosin seed specific promoter from
21 *Brassica napus* or *Arabidopsis thaliana* (van Rooijen and Moloney, 1994). Promoter
22 selection is also dependent upon the desired efficiency and level of peptide or
23 protein production. Inducible promoters such *tac* and *aox1* are often employed in
24 order to dramatically increase the level of protein expression. Overexpression of
25 proteins may be harmful to the host cells. Consequently, host cell growth may be
26 limited. The use of inducible promoter systems allows the host cells to be cultivated
27 to acceptable densities prior to induction of gene expression, thereby facilitating
28 higher product yields. If the protein coding sequence is to be integrated through a
29 gene replacement (omega insertion) event into a target locus, then promoter
30 selection may also be influenced by the degree of homology to the target locus
31 promoter.

1 Various signal sequences may be used according to the present invention.
2 A signal sequence which is homologous to the protein coding sequence to be
3 expressed may be used. Alternatively, a signal sequence which has been selected
4 or designed for improved secretion in the expression host may also be used. For
5 example, *B. subtilis* *sacB* signal sequence for secretion in *B. subtilis*, the
6 *Saccharomyces cerevisiae* α -mating factor or *P. pastoris* acid phosphatase *phoA*
7 signal sequences for *P. pastoris* secretion may be used. A signal sequence with a
8 high degree of homology to the target locus may be required if the protein coding
9 sequence is to be integrated through an omega insertion event. The signal
10 sequence may be joined directly through the sequence encoding the signal
11 peptidase cleavage site to the protein coding sequence, or through a short
12 nucleotide bridge consisting of usually fewer than ten codons.

13 Elements for enhancing expression transcription (promoter activity) and
14 translation have been identified for eukaryotic protein expression systems. For
15 example, positioning the cauliflower mosaic virus (CaMV) promoter 1000 bp on either
16 side of a heterologous promoter may elevate transcriptional levels by 10- to 400-fold.
17 The expression construct should also include the appropriate translational initiation
18 sequences. Modification of the expression construct to include the Kozak consensus
19 sequence for proper translational initiation may increase the level of translation by
20 10 fold.

21 Elements to enhance purification of the protein may also be included in the
22 expression construct. The product of oleosin gene fusions is a hybrid protein
23 containing the oleosin gene joined to the gene product of interest. The fusion protein
24 retains the lipophilic properties of oleosins and is incorporated in the oil body
25 membranes (van Rooijen and Moloney, 1994). Association with the oil bodies may
26 be exploited to facilitate purification of the recombinant oleosin fusion proteins (van
27 Rooijen and Moloney, 1994).

28 A selection marker is usually employed, which may be part of the expression
29 construct or separate from it (e.g., carried by the expression vector), so that the
30 marker may integrate at a site different from the gene of interest. Transformation of
31 the host cells with the recombinant DNA molecules of the invention is monitored
32 through the use of selectable markers. Examples of these are markers that confer

1 resistance to antibiotics (e.g., *bla* confers resistance to ampicillin for *E. coli* host cells,
2 *nptII* confers kanamycin resistance to *B. napus* cells) or that permit the host to grow
3 on minimal medium (e.g., *HIS4* enables *P. pastoris* GS115 His⁻ to grow in the
4 absence of histidine). The selectable marker will have its own transcriptional and
5 translational initiation and termination regulatory regions to allow for independent
6 expression of the marker. Where antibiotic resistance is employed as a marker, the
7 concentration of the antibiotic for selection will vary depending upon the antibiotic,
8 generally ranging from 10 to 600 µg of the antibiotic/mL of medium.

9 The expression construct is assembled by employing known recombinant
10 DNA techniques. Restriction enzyme digestion and ligation are the basic steps
11 employed to join two fragments of DNA. The ends of the DNA fragment may require
12 modification prior to ligation and this may be accomplished by filling in overhangs,
13 deleting terminal portions of the fragment(s) with nucleases (e.g., *ExoIII*), site
14 directed mutagenesis, and adding new base pairs by the polymerase chain reaction
15 (PCR). Polylinkers and adaptors may be employed to facilitate joining of select
16 fragments. The expression construct is typically assembled in stages employing
17 rounds of restriction, ligation and transformation of *E. coli*. There are numerous
18 cloning vectors available for construction of the expression construct and the
19 particular choice is not critical to this invention. The selection of cloning vector will
20 be influenced by the gene transfer system selected for introduction of the expression
21 construct into the host cell. At the end of each stage, the resulting construct may be
22 analyzed by restriction, DNA sequence, hybridization and PCR analyses.

23 The expression construct may be transformed into the host as the cloning
24 vector construct, either linear or circular, or may be removed from the cloning vector
25 and used as is or introduced onto a delivery vector. The delivery vector facilitates
26 the introduction and maintenance of the expression construct in the selected host
27 cell type. The expression construct is introduced into the host cells by employing any
28 of a number of gene transfer systems (e.g., natural competence, chemically
29 mediated transformation, protoplast transformation, electroporation, biolistic
30 transformation, transfection, or conjugation). The gene transfer system selected
31 depends upon the host cells and vector systems used.

1 For instance, the expression construct can be introduced into *P. pastoris* cells
2 by protoplast transformation or electroporation. Electroporation of *P. pastoris* is
3 easily accomplished and yields transformation efficiencies comparable to spheroplast
4 transformation. *P. pastoris* cells are washed with sterile water and resuspended in
5 a low conductivity solution (e.g., 1 M sorbitol solution). A high voltage shock applied
6 to the cell suspension creates transient pores in the cell membrane through which
7 the transforming DNA (e.g., expression construct) enters the cells. The expression
8 construct is stably maintained by integration, through homologous recombination,
9 into the *aox1* (alcohol oxidase) locus.

10 Alternatively, an expression construct, comprising the *sacB* promoter and
11 signal sequence operably linked to the protein coding sequence, is carried on
12 pUB110, a plasmid capable of autonomously replicating in *B. subtilis* cells. The
13 resulting plasmid construct is introduced into *B. subtilis* cells by transformation.
14 *Bacillus subtilis* cells develop natural competence when grown under nutrient poor
15 conditions.

16 In a third example, *Brassica napus* cells are transformed by *Agrobacterium*-
17 mediated transformation. The expression construct is inserted onto a binary vector
18 capable of replication in *A. tumefaciens* and mobilization into plant cells. The
19 resulting construct is transformed into *A. tumefaciens* cells carrying an attenuated Ti
20 or "helper plasmid". When leaf disks are infected with the recombinant *A.*
21 *tumefaciens* cells, the expression construct is transferred into *B. napus* leaf cells by
22 conjugal mobilization of the binary vector::expression construct. The expression
23 construct integrates at random into the plant cell genome.

24 Host cells carrying the expression construct (i.e., transformed cells) are
25 identified through the use of the selectable marker carried by the expression
26 construct or vector and the presence of the gene of interest confirmed by a variety
27 of techniques including hybridization, PCR, and antibodies.

28 The transformant microbial cells may be grown by a variety of techniques
29 including batch and continuous fermentation on liquid or semi-solid media.
30 Transformed cells are propagated under conditions optimized for maximal product-
31 to-cost ratios. Product yields may be dramatically increased by manipulating of
32 cultivation parameters such as temperature, pH, aeration, and media composition.

1 Careful manipulation and monitoring of the growth conditions for recombinant hyper-
2 expressing *E. coli* cells may result in culture biomass and protein yields of 150 g (wet
3 weight) of cells/L and 5 g of insoluble protein/L, respectively. Low concentrations of
4 a protease inhibitor (e.g., phenylmethylsulfonyl fluoride or pepstatin) may be
5 employed to reduce proteolysis of the over-expressed peptide or protein.
6 Alternatively, protease deficient host cells may be employed to reduce or eliminate
7 degradation of the desired protein.

8 After selection and screening, transformed plant cells can be regenerated into
9 whole plants and varietal lines of transgenic plants developed and cultivated using
10 known methods. As used herein, "transgenic plant" includes transgenic plants, plant
11 tissues and plant cells.

12 Following fermentation, the microbial cells may be removed from the medium
13 through down-stream processes such as centrifugation and filtration. If the desired
14 product is secreted, it can be extracted from the nutrient medium. In the case of
15 intracellular production, the cells are harvested and the product released by rupturing
16 cells through the application of mechanical forces, ultrasound, enzymes, chemicals
17 and/or high pressure. Production of an insoluble product, such as occurs in hyper-
18 expressing *E. coli* systems, can be used to facilitate product purification. The
19 product inclusions can be extracted from disrupted cells by centrifugation and
20 contaminating proteins may be removed by washing with a buffer containing low
21 concentrations of a denaturant (e.g., 0.5 to 6 M urea, 0.1 to 1% sodium dodecyl
22 sulfate or 0.5 to 4.0 M guanidine-HCl). The washed inclusions may be solubilized
23 in solutions containing 6 to 8 M urea, 1 to 2% sodium dodecyl sulfate or 4 to 6 M
24 guanidine-HCl. Solubilized product can be renatured by slowly removing denaturing
25 agents during dialysis.

26 Phytase may be extracted from harvested portions or whole plants by
27 grinding, homogenization, and/or chemical treatment. The use of seed specific
28 lipophilic oleosin fusions can facilitate purification by partitioning the oleosin fusion
29 protein in the oil fraction of crushed canola seeds, away from the aqueous proteins
30 (van Rooijen and Moloney, 1994).

31 If necessary, various methods for purifying the product, from microbial,
32 fermentation and plant extracts, may be employed. These include precipitation (e.g.,

1 ammonium sulfate precipitation), chromatography (gel filtration, ion exchange, affinity
2 liquid chromatography), ultrafiltration, electrophoresis, solvent-solvent extraction
3 (e.g., acetone precipitation), combinations thereof, or the like.

4 All or a portion of the microbial cultures and plants may be used directly in
5 applications requiring the action of phytase. Various formulations of the crude or
6 purified phytase preparations may also be prepared. The enzymes can be stabilized
7 through the addition of other proteins (e.g., gelatin, skim milk powder) and chemical
8 agents (e.g., glycerol, polyethylene glycol, reducing agents and aldehydes). Enzyme
9 suspensions can be concentrated (e.g., tangential flow filtration) or dried (spray and
10 drum drying, lyophilization) and formulated as liquids, powders, granules, pills,
11 mineral blocks and gels through known processes. Gelling agents such as gelatin,
12 alginate, collagen, agar, pectin and carrageenan may be used.

13 Further, complete dephosphorylation of phytate may not be achieved by
14 phytase alone. Phytases may not dephosphorylate the lower *myo*-inositol
15 phosphates. For instance, an *A. ficuum* phytase described in U.S. Patent No.
16 5,536,156 (van Gorcum *et. al.*, issued July 25, 1995) exhibits low or no phosphatase
17 activity against *myo*-inositol di-phosphate or *myo*-inositol mono-phosphate. Addition
18 of another phosphatase, such as an acid phosphatase, to a feed additive of the
19 present invention containing phytase will help dephosphorylate *myo*-inositol
20 di-phosphate and *myo*-inositol mono-phosphate.

21 Formulations of the desired product may be used directly in applications
22 requiring the action of a phytase. Liquid concentrates, powders and granules may
23 be added directly to reaction mixtures, fermentations, steeping grains, and milling
24 waste. The formulated phytase can be administered to animals in drinking water, in
25 a mineral block, as a salt, or as a powdered supplement to be sprinkled into feed
26 bunks or mixed with a ration. It may also be mixed with, sprayed on or pelleted with
27 other feed stuffs through known processes. Alternatively, a phytase gene with a
28 suitable promoter-enhancer sequence may be intergrated into an animal genome
29 and selectively expressed in an organ or tissue (e.g. salivary glands, pancreas or
30 epithelial cells) which secrete the phytase enzyme into the gastrointestinal tract,
31 thereby eliminating the need for the addition of supplemental phytase.

1 In a preferred formulation, phytases of the present invention may take the
2 form of microbial feed inoculants. Cultures of microorganisms expressing a native
3 phytase, such as *S. ruminantium* JY35 (ATCC 55785), or recombinant
4 microorganisms expressing a phytase encoded by a heterologous phytase gene are
5 grown to high concentrations in fermenters and then harvested and concentrated by
6 centrifugation. Food-grade whey and/or other cryoprotective agents are then
7 admixed with the cell concentrate. The resulting mixture is then cryogenically frozen
8 and freeze-dried to preserve phytase activity by standard lyophilization procedures.
9 The freeze-dried culture may be further processed to form a finished product by such
10 further steps as blending the culture with an inert carrier to adjust the strength of the
11 product.

12 All or a portion of the microbial cultures and plants as produced by the present
13 invention may be used in a variety of industrial processes requiring the action of a
14 phytase. Such applications include, without limitation, the manufacture of end
15 products such as inositol phosphate and inositol, production of feed ingredients and
16 feed additives for non-ruminants (e.g., swine, poultry, fish, pet food), in human
17 nutrition, and in other industries (soybean and corn processing, starch, and
18 fermentation) that involve feedstocks containing phytate. Degradation of phytate
19 makes inorganic phosphate and chelated metals available to animals and
20 microorganisms. The action of phytase increases the quality, value and utility of feed
21 ingredients and/or fermentation substrates that are high in phytate. The action of
22 phytases can also accelerate the steeping process and separation processes
23 involved in the wet milling of corn.

24 The phytase genes of the present invention can be used in heterologous
25 hybridization and polymerase chain reaction experiments, directed to isolation of
26 phytase encoding genes from other microorganisms. The examples herein are given
27 by way of illustration and are in no way intended to limit the scope of the present
28 invention. Efforts have been made to ensure the accuracy with respect to numbers
29 used (e.g., temperature, pH, amounts) but the possibility of some experimental
30 variance and deviations should be recognized.

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Example 1

Isolation of ruminal bacteria

Ruminal fluid from a cannulated Holstein cow was collected in a sterile Whirlpak™ bag. Fluid may also be withdrawn from the rumen via an orogastric tube. Under a suitable anaerobic atmosphere (e.g., 90% CO₂ and 10% H₂), ten-fold serial dilutions of the rumen fluid were prepared and distributed over the surface of a solid growth medium (e.g., Scott and Dehority, 1965), and the plates were incubated at 39°C for 18 to 72 h. Isolated colonies were picked with a sterile loop and the cells were spread over the surface of fresh agar medium to produce isolated colonies. The cells from a single colony were confirmed by morphological examination to represent a pure culture and were cultured and stored in the Lethbridge Research Centre ("LRC") culture collection or used as a source of enzymatic activity or genetic material.

Example 2

Screening ruminal bacteria for phytase activity

A. Phytase assays

Sample solutions (culture filtrates, cell suspensions, lysates, washes or distilled water blanks) were assayed for phytase activity by incubating 150 µl of the solution with 600 µl of substrate solution [0.2% (w/v) sodium phytate in 0.1 M sodium acetate buffer, pH 5.0] for 30 min at 37°C. The reaction was stopped by adding 750 µl of 5% (w/v) trichloroacetic acid. Released orthophosphate in the reaction mixture was measured by the method of Fiske and Subbarow (1925). Freshly prepared colour reagent [750 µl of a solution containing 4 volumes of 1.5% (w/v) ammonium molybdate in a 5.5% (v/v) sulfuric acid solution and 1 volume of a 2.7% (w/v) ferrous sulfate solution] was added to the reaction mixture and the production of phosphomolybdate was measured spectrophotometrically at 700 nm. Results were compared to a standard curve prepared with inorganic phosphate. One unit ("Unit") of phytase was defined as the amount of enzyme required to release one µmole of inorganic phosphate (P_i) per min under the assay conditions.

An improved phytase plate assay was developed which eliminated false positive results caused by microbial acid production. Bacterial isolates were grown

1 under anaerobic conditions on modified Scott and Dehority (1965) agar medium
2 containing 5% (v/v) rumen fluid, 1.8% (w/v) agar and 2.0% (w/v) sodium phytate for
3 5 d at 37°C. Colonies were washed from the agar surface and the petri plates were
4 flooded with a 2% (w/v) aqueous cobalt chloride solution. After a 5-min incubation
5 at room temperature the cobalt chloride solution was replaced with a freshly
6 prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium
7 molybdate solution and 0.42% (w/v) ammonium vanadate solution. Following a 5-
8 min incubation, the ammonium molybdate solution/ammonium vanadate solution was
9 removed and the plates examined for zones of clearing. The effectiveness of this
10 counterstaining technique is demonstrated in Figure 1. Prior to staining, zones of
11 clearing were evident around colonies of phytase-producing *S. ruminantium* JY35
12 (ATCC 55785) and lactic acid-producing *S. bovis* grown on agar medium containing
13 phytate (Figure 1, left petri plate). The false positive zones of clearing resulting from
14 acid production by *S. bovis* colonies were eliminated by counterstaining the plates
15 with cobalt chloride and ammonium molybdate/ammonium vanadate solutions
16 (Figure 1, right petri plate).

18 B. Phytase activity of ruminal bacteria

19 The phytase activities of 345 rumen bacteria from the LRC culture collection
20 were determined (Table 1). The anaerobic technique of Hungate (1950), as modified
21 by Bryant and Burkey (1953), or an anaerobic chamber with a 90% CO₂ and
22 10% H₂ atmosphere was used to cultivate the microorganisms in the LRC culture
23 collection. Phytase screening was performed on isolates grown anaerobically (100%
24 CO₂) in Hungate tubes with 5 mL of modified Scott and Dehority medium (1965)
25 containing 5% (v/v) rumen fluid, 0.2% (w/v) glucose, 0.2% (w/v) cellobiose and 0.3%
26 (w/v) starch. After 18 to 24 h incubation at 39°C, whole cells or culture supernatants
27 were assayed for phytase activity. Selenomonads were the predominant phytase
28 producers (93% of the isolates tested had phytase activity, Table 1). *Prevotella* was
29 the only other genus from which a significant number of positive cultures was
30 identified (11 phytase positive isolates out of 40 tested). A total of 29 cultures with
31 substantial phytase activity were identified. These included 24 of the genus
32 *Selenomonas* and 5 of the genus *Prevotella*. Twelve of these cultures (11

1 *Selenomonas* and 1 *Prevotella* isolate) had phytase activities substantially higher
2 than the other positive cultures (Table 2). In all instances, the phytase activity was
3 predominantly cell associated.

4 5 6 Example 3

7 Phytase activity of *Selenomonas ruminantium* JY35 (ATCC 55785)

8 A. Growth and phytase production

9 Phytase production during growth of *S. ruminantium* JY35 (ATCC 55785) was
10 examined. *S. ruminantium* JY35 (ATCC 55785) was grown at 39°C in Hungate tubes
11 with 5 mL of modified Scott and Dehority broth (1965) containing 5% (v/v) ruminal
12 fluid. Growth (protein concentration) and phytase activity (cell associated) were
13 monitored at intervals over a 24-h time period. Maximal growth and phytase activity
14 of *S. ruminantium* JY35 (ATCC 55785) were achieved 8-10 h after inoculation
15 (Figure 2). Cell growth was mirrored by increases in phytase activity.

16 17 B. Localization of phytase activity

18 *S. ruminantium* JY35 (ATCC 55785) phytase activity was determined to be
19 predominantly cell associated. Little phytase activity was detected in culture
20 supernatants and cell washes. The phytase activity of *S. ruminantium* JY35 (ATCC
21 55785) was localized by electron microscopy as described by Cheng and Costerton
22 (1973). Cells were harvested by centrifugation, washed with buffer, embedded in 4%
23 (w/v) agar, prefixed in 0.5% glutaraldehyde solution for 30 min and fixed for 2 hours
24 in 5% (v/v) glutaraldehyde solution. Samples were washed five times with
25 cacodylate buffer (0.1 M, pH 7.2) and treated with 2% (w/v) osmium tetroxide,
26 washed five times with cacodylate buffer, dehydrated in a graded ethanol series, and
27 embedded in Spurr's resin (J. B. EM Services Inc.). Ultrathin sections were cut with
28 a Reichert model OM U3 ultramicrotome and stained with 2% (w/v) uranyl acetate
29 and lead citrate. Specimens were viewed with Hitachi H-500 TEM at an accelerating
30 voltage of 75 kV. A comparison of *S. ruminantium* JY35 (ATCC 55785) cells
31 incubated with substrate for reaction product deposition with untreated cells clearly
32 indicated that the phytase activity was associated with the cell outer membrane

1 surfaces (Figure 3). Deposition of electron dense material on the outer cell surfaces
2 of treated cells was the result of phytase activity (Figures 3A, B and C).

3
4 C. Phytase pH optimum

5 Initial determinations of the pH optimum of the *S. ruminantium* JY35 (ATCC
6 55785) phytase were conducted with whole cells. Phytase activity was optimal over
7 a pH range of 4.0 to 5.5 (Figure 4). A second pH curve was generated with a MgCl_2
8 cell extract (Figure 5). Cells from a 100-mL overnight culture were washed twice with
9 sterile distilled water, resuspended in 0.3 volumes of a 0.2 M MgCl_2 aqueous solution
10 and incubated overnight at 0°C. The solution was clarified by centrifugation and the
11 resulting extract was used in phytase assays. Four buffers systems were used to
12 cover the pH range; glycine (pH 1.5 - 3.0), formate (pH 3.0 - 4.0), acetate (pH 4.0 -
13 5.5) and succinate (pH 5.5 - 6.5).

14
15 D. Phytase temperature optimum

16 The temperature optimum of the *S. ruminantium* JY35 (ATCC 55785) phytase
17 activity was determined at pH 5.0 (0.1 M sodium acetate buffer) with MgCl_2 cell
18 extract. The enzyme retained over 50% of its activity over a temperature range of
19 37 to 55°C (Figure 6).

20
21 E. The effect of ions and substrate concentration on phytase activity

22 The effect of various ions (10 mM) and substrate concentration on whole cell
23 phytase activity were determined at pH 5.0 (0.1 M sodium acetate buffer). Phytase
24 activity was stimulated by the addition of Ca^{++} , Na^+ , K^+ and Mg^{++} , inhibited by Fe^{++} ,
25 Zn^{++} and Mn^{++} and unaffected by Co^{++} and Ni^{++} (Figure 7). The effect of substrate
26 concentration on phytase activity in a *S. ruminantium* JY35 (ATCC 55785) MgCl_2
27 cell extract is presented in Figure 8.

28
29 F. Molecular Weight

30 The molecular size of the phytase in *S. ruminantium* JY35 (ATCC 55785) was
31 determined by zymogram analysis. A ten-fold concentrated crude MgCl_2 released
32 extract was mixed with 20 μL of sample loading buffer (Laemmli, 1970) in a

1 microtube and the microtube was placed in a boiling water bath for 5 minutes. The
2 denatured MgCl_2 extracts were resolved by SDS-PAGE on a 10% separating gel
3 topped with a 4% stacking gel (Laemmli, 1970). Following electrophoresis, the
4 phytase was renatured by soaking the gel in 1% Triton X-100 for 1 h at room
5 temperature and 0.1 M sodium acetate buffer (pH 5.0) for 1 h at 4°C. Phytase
6 activity was detected by incubating the gel for 16 h in a 0.1 M sodium acetate buffer
7 (pH 5.0) containing 0.4% sodium phytate. The gel was treated with the cobalt
8 chloride and ammonium molybdate/ammonium vanadate staining procedure
9 described for the phytase plate assays in Example 2. A single dominant activity
10 band, corresponding to a molecular mass of approximately 35 to 45 kDa, was
11 observed (Figure 9).

12 13 Example 4

14 Cloning of a phytase gene (*phyA*) from *Selenomonas ruminantium* JY35 (ATCC 15 55785)

16 A. Isolation of phytase positive *Escherichia coli* clone

17 Genomic DNA libraries were prepared for *S. ruminantium* JY35 (ATCC 55785)
18 according to published procedures (Hu *et al.*, 1991; Sambrook *et al.*, 1989).
19 Genomic DNA was extracted from a fresh overnight culture of *S. ruminantium* JY35
20 (ATCC 55785) using a modification of the protocol described by Priefer *et al.* (1984).
21 *S. ruminantium* JY35 (ATCC 55785) genomic DNA was partially digested with *Sau*3A
22 and gel purified to produce DNA fragments in the 2- to 10-kb range. A genomic
23 library was constructed by ligating *Bam*HI-digested, dephosphorylated pUC18 with
24 *S. ruminantium* JY35 (ATCC 55785) *Sau*3A genomic DNA fragments. *Escherichia*
25 *coli* DH5 α competent cells (Gibco BRL, Mississauga, ON) were transformed with the
26 ligation mix and 6,000 clones carrying inserts were screened for phytase activity
27 (zones of clearing) on LB phytase screening agar [LB medium, 1.0 % sodium phytate
28 (filter sterilized), 100 mM HEPES (pH 6.0 - 6.5), and 0.2 % CaCl_2] containing
29 ampicillin (100 $\mu\text{g/mL}$). A phytase-positive clone SrP.2 was isolated and phytase
30 activity confirmed through enzyme assays (Figure 10). Very high levels of phytase
31 activity were found in the medium as well as associated with the *E. coli* cells (Table

1 3). Plasmid DNA isolated from clone SrP.2 carried a 5.5-kb plasmid, designated
2 pSrP.2, consisting of pUC18 containing a 2.7-kb *Sau3A* insert.

3
4 B. Confirmation of the *Selenomonas ruminantium* JY35 (ATCC 55785) origin of
5 the 2.7-kb insert

6 The *S. ruminantium* JY35 (ATCC 55785) origin of the 2.7-kb insert in pSrP.2
7 was confirmed by Southern blot hybridization (Sambrook *et al.*, 1989). Genomic
8 DNA isolated from *S. ruminantium* JY35 (ATCC 55785) and digested with *EcoRI* or
9 *HindIII* was resolved on a 0.8% agarose gel. After transfer to Zeta-probe[®] membrane
10 (BioRad Laboratories), the hybridization was performed overnight at high stringency
11 (2 x SSC; 65°C) with the 2.7-kb fragment from pSrP.2 labelled with digoxigenin (DIG
12 DNA labeling and detection kit; Boehringer Mannheim Canada Ltd., Laval, PQ). The
13 blots were washed twice in 2 x SSC at room temperature; 0.1% SDS for 5 minutes
14 and twice 0.1 x SSC; 0.1% SDS for 20 minutes at 65°C. The blots were developed
15 according to the protocol provided with the DIG DNA labeling and detection kit
16 (Boehringer Mannheim Canada Ltd).

17 The probe reacted with a 14-kb *HindIII* (Figure 11) and a 23-kb *EcoRI* (data
18 not shown) fragment of genomic DNA and confirmed that the 2.7-kb fragment was
19 from *S. ruminantium* JY35 (ATCC 55785) and that a single homologous sequence
20 exists in the genome. Single copies of a sequence homologous to the 2.7-kb
21 fragment from *S. ruminantium* JY35 (ATCC 55785) also exist in the genomes of *S.*
22 *ruminantium* HD86, HD141, and HD₄ (data not shown). However restriction fragment
23 length polymorphisms were noted for *S. ruminantium* HD86 (9- and 23-kb *EcoRI*
24 fragments) and *S. ruminantium* HD₄ (3-kb *EcoRI* fragment and a 20-kb *HindIII*
25 fragment). The labelled 2.7-kb fragment from pSrP.2 failed to hybridize with genomic
26 DNA isolated from *Prevotella* sp. 46/5², *E. coli* DH5α or *A. ficuum* NRRL 3135 (data
27 not shown).

Example 5

Characterization of *Selenomonas ruminantium* phytase gene

A. Evidence for the cloning of a phytase gene

Escherichia coli DH5 α competent cells (Gibco BRL, Mississauga, ON) were transformed with plasmids pUC18 and pSrP.2. The resulting ampicillin-resistant transformants were tested for phytase activity on LB phytase screening agar. Only *E. coli* DH5 α cells transformed with pSrP.2 produced clearing zones on LB phytase screening agar.

B. Restriction and deletion analysis of pSrP.2

The phytase gene was localized on the 2.7-kb *Sau*3A insert by restriction endonuclease and deletion analyses (Ausubel *et al.*, 1990; Sambrook *et al.*, 1989). Cells carrying plasmid pSrP.2 Δ *Sph*I, constructed by the deletion of the 1.4-kb *Sph*I fragment from pSrP.2, lacked phytase activity (Figure 12 and Figure 13, Table 3).

C. Zymogram analysis

The molecular mass of the phytase produced by *E. coli* DH5 α (pSrP.2) was determined by zymogram analysis. One mL of an overnight culture was transferred to a 1.5-mL microtube. The cells were harvested by centrifugation and washed with 0.1 M sodium acetate buffer (pH 5.5). The cell pellet was resuspended in 80 μ L of sample loading buffer (Laemmli, 1970) and the microtube was placed in a boiling water bath for 5 minutes. The resulting cell extracts were resolved by SDS-PAGE on a 10% separating gel topped with a 4% stacking gel (Laemmli, 1970) and the gel was stained for phytase activity as described in Example 3F. A single dominant activity band, corresponding to a molecular mass of approximately 37 kDa, was observed (Figure 14, lane A). A corresponding activity band was not observed for *E. coli* DH5 α (pSrP.2 Δ *Sph*I) cells (Figure 14, lane B).

D. DNA sequence analysis of pSrP.2

The complete sequence of the 2.7-kb insert of pSrP.2 was determined. Samples were prepared for DNA sequence analysis on an Applied Biosystems Model 373A DNA sequencing system (Applied Biosystems, Inc., Mississauga, ON)

1 by using a Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems,
2 Inc.). Template DNA was extracted from overnight cultures of *E. coli* DH5α (pSrP.2)
3 with the Wizards™ minipreps DNA purification system (Promega Corp., Madison,
4 WI). Overlapping sequences were generated by primer walking. The DNA
5 sequence data was analyzed using MacDNASIS DNA software (Hitachi Software
6 Engineering Co., Ltd., San Bruno, CA).

7 The sequence of the 2.7-kb DNA insert was determined and DNA structural
8 analysis identified an open reading frame (ORF2; bp 1493 to 2504) overlapping the
9 *SphI* site of the 2.7-kb *Sau3A* insert and large enough to encode the 37 kDa phytase.
10 Phytase activity was eliminated by deleting bp 1518 through to the end of the 2.7-kb
11 *Sau3A* fragment (pSrPr6, Table 3, Figure 13). This was accomplished by cloning the
12 PCR product of pSrP.2 bounded by sequencing primer SrPr6 (CGG GAT GCT TCT
13 GCC AGT AT, SEQ ID NO. 3 the reverse complement of bp 1518 to 1538) and M13
14 Forward primer (CGC CAG GGT TTT CCC AGT CAC GAC) into pGEM-T (Promega
15 Corp.). A PCR product subclone (pSrPf6) of pSrP.2, bounded by primer SrPf6 (bp
16 1232 to 1252, CGT CCA CGG AGT CAC CCT AC) SEQ ID NO. 4 and M13 Reverse
17 primer (AGC GGA TAA CAA TTT CAC ACA GGA), and containing ORF2 plus 252
18 bp upstream of the *SphI* cleavage site retained phytase activity (Table 3, Figure 13).

19 The sequence and translation of the *S. ruminantium* phytase gene (*phyA*) is
20 shown in Figure 15. Translation of ORF2 would result in the expression of a 346-
21 amino acid polypeptide with a predicted molecular weight of 39.6 kDa (Figure 15).
22 The first 31 residues were typical of a prokaryote signal sequence, encompassing
23 a basic N-terminus and central hydrophobic core (von Heijne, 1986). Application of
24 the method of von Heijne (1986) predicted the signal peptidase cleavage site most
25 probably occurs before Ala²⁸ or Pro³¹. This was confirmed by determining the N-
26 terminal amino acid sequence of gel purified from *E. coli* DH5α (pSrPf6) culture
27 supernatant (Figure 15). The secreted mature protein has a putative mass of 36.5
28 kDa.

29 A comparison of the *phyA* amino acid sequence with known protein
30 sequences from the MasDNASIS SWISSPROT database revealed no significant
31 similarities to any published sequences including *Aspergillus niger* phytase genes
32 *phyA* and *phyB*.

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Cell free supernatants, prepared from overnight cultures of *E. coli* (pSrPf6), were mixed 3:1 (v/v) with Ni²⁺-NTA agarose pre-equilibrated in 0.1 M Tris (pH 7.9), 0.3 M NaCl buffer. The mixture was incubated at room temperature for 0.5 h and washed 3 x with 0.1 M Tris (pH 7.9), 0.3 M NaCl buffer. The phytase activity was eluted from the resin with 1 volume 0.1 M sodium acetate (pH 5.0), 0.3 M NaCl. When resolved on SDS-polyacrylamide gels stained with Coomassie brilliant blue, over 70% of the eluted protein formed a single 37-kDa protein band. Zymogram and N-terminal amino acid sequence analyses confirmed that the 37-kDa band corresponded to the phytase encoded by the cloned *S. ruminantium* JY35 (ATCC 55785) *phyA*. The specific activity of Ni²⁺-NTA agarose-purified phytase ranged from 200 to 400 μ mol phosphate released/min/mg protein. This is 2 to 4 times higher than the specific activity reported for the purified *A. ficuum* NRRL 3135 phytase (van Gorcum et al., 1991, 1995; van Hartingsveldt et al., 1993).

Overexpression of the *Selenomonas ruminantium* phyA gene

Isolation and characterization of *phyA* from *S. ruminantium* JY35 (ATCC 55785) enables the large scale production of protein PhyA in any of a number of prokaryotic (e.g., *E. coli* and *B. subtilis*) or eukaryotic (e.g., fungal - *Pichia*, *Saccharomyces*, *Aspergillus*, *Trichoderma*; plant - *Brassica*, *Zea*, *Solanum*; or animal - poultry, swine or fish) expression systems using known methods. Teachings for the construction and expression of *phyA* in *E. coli*, *P. pastoris*, and *B. napus* are provided below. Similar approaches may be adopted for expression of the *S. ruminantium* JY35 (ATCC 55785) phytase in other prokaryotic and eukaryotic organisms.

1 A. Cloning of the *Selenomonas ruminantium* *phyA* in an *Escherichia coli* - specific
2 expression construct

3 An expression construct is constructed in which the region encoding the
4 mature *PhyA* is transcriptionally fused with the *tac* promoter (Brosius et al., 1985).
5 The promoter sequences may be replaced by those from other promoters that
6 provide for efficient expression in *E. coli*. The expression construct is introduced into
7 *E. coli* cells by transformation.

8 i. Construction of the *E. coli* expression vector

9 A number of *E. coli* expression vectors based on the *tac* or related promoters
10 are commercially available. In this example the construct will be prepared with
11 pKK223-3 available from Pharmacia Biotech Inc. (Uppsala, Sweden). The region of
12 *phyA* encoding the mature *PhyA* (the peptide secreted following removal of the
13 signal peptide) is amplified with oligonucleotide primers MATE2 (GC GAA TTC ATG
14 GCC AAG GCG CCG GAG CAG AC) (SEQ ID NO. 5) and M13 Reverse. The
15 oligonucleotide MATE2 (SEQ ID NO. 5) was designed to contain a suitable restriction
16 site at its terminus to allow direct assembly of the amplified product with pKK223-3.
17 The region of *phyA* amplified with MATE2 (SEQ ID NO. 5) and M13 Reverse is
18 digested with *EcoRI* and *SmaI* and ligated into similarly cleaved pKK223-3.

19 ii. Transformation of *E. coli* and *PhyA* expression

20 The pKK223-3::*phyA* ligation mix is used to transform competent *E. coli* cells.
21 Strains suitable for high levels of protein expression, such as SG13009, CAG926 or
22 CAG929 (carrying *lacI* on a plasmid such as pREP4), are employed. Transformed
23 cells are spread on LB agar containing ampicillin (100 µg/mL) and incubated
24 overnight at 37°C. Ampicillin-resistant colonies are screened for the presence of the
25 desired pKK223-3::*phyA* construct by extracting pDNA and subjecting the pDNA to
26 agarose gel electrophoresis and restriction analysis. Positive clones may be further
27 characterized by PCR and DNA sequence analysis.

28 Expression of the *S. ruminantium* JY35 (ATCC 55785) phytase by
29 transformed *E. coli* cells is tested by growing the cells under vigorous aeration at
30 37°C in a suitable liquid medium (e.g., LB or 2xYT) containing the appropriate
31 antibiotic selection until the optical density (at 600 nm) is between 0.5 and 1.0. The
32 *tac* promoter is induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final

1 concentration between 0.1 and 2 mM. The cells are cultivated for an additional 2 to
2 4 h and harvested by centrifugation. Protein expression is monitored by SDS-PAGE,
3 and western blot/immunodetection techniques. The expressed PhyA may be
4 extracted by breaking (e.g., sonication or mechanical disruption) the *E. coli* cells.
5 Protein inclusions of PhyA may be harvested by centrifugation and solubilized with
6 1 to 2 % SDS. The SDS may be removed by dialysis, electroelution or ultrafiltration.
7 The phytase activity of prepared cell extracts may be assayed by standard methods
8 described in Example 2.

9
10 B. Cloning of the *Selenomonas ruminantium* phyA in a *Pichia pastoris* - specific
11 expression construct

12 An expression construct is constructed in which the region encoding the
13 mature PhyA is translationally fused with the secretion signal sequences found on
14 *P. pastoris* expression vectors (Pichia Expression Kit Instruction Manual, Invitrogen
15 Corporation, San Diego, CA) in order to express the *S. ruminantium* phytase as a
16 secreted product. The promoter and secretion signal sequences may be replaced
17 by those from other promoters that provide for efficient expression in *Pichia*. The
18 expression construct is introduced into *P. pastoris* cells by transformation.

19 i. Construction of the *P. pastoris* expression vector

20 A number of *P. pastoris* expression vectors based on the *aox1* promoters and
21 α -Factor or *pho1* signal sequences are commercially available. In this example the
22 construct will be prepared with pPIC9 available from Invitrogen Corporation. The
23 region of *phyA* encoding the mature PhyA is amplified with oligonucleotide primers
24 MATE (GC GAA TTC GCC AAG GCG CCG GAG CAG AC) (SEQ ID NO. 6) and
25 M13 Reverse. The oligo MATE (SEQ ID NO. 6) was designed to contain a suitable
26 restriction site at its terminus to allow direct assembly of the amplified product with
27 pPIC9. The region of *phyA* amplified with MATE (SEQ ID NO. 6) and M13 Reverse
28 is digested with *EcoRI* and ligated into similarly cleaved pPIC9.

29 ii. Transformation of *P. pastoris* and PhyA expression

30 The pPIC9::*phyA* ligation mix is used to transform competent *E. coli* DH5 α
31 cells. Transformed cells are spread on LB agar containing ampicillin (100 μ g/mL)
32 and incubated overnight at 37°C. Ampicillin-resistant colonies are screened for the

1 presence of the desired pPIC9::*phyA* construct by extracting pDNA and subjecting
2 the pDNA to agarose gel electrophoresis and restriction analysis. Positive clones are
3 further characterized by PCR and DNA sequence analysis. Plasmid DNA is
4 prepared from a 1 L culture of an *E. coli* clone carrying the desired pPIC9::*phyA*
5 construct. The pDNA is digested with *Bgl*I and analyzed by agarose gel
6 electrophoresis to confirm complete digestion of the vector. The digested pDNA is
7 extracted with phenol:chloroform, ethanol precipitated and resuspended in sterile
8 distilled H₂O to a final concentration of 1 µg/mL. In preparation for transformation,
9 *P. pastoris* GS115 or KM71 cells are grown for 24 h at 30°C in YPD broth. Cells from
10 100 µL of culture are harvested by centrifugation and resuspended in 100 µL of
11 transformation buffer (0.1M LiCl, 0.1M dithiothreitol, 45% polyethylene glycol 4000)
12 containing 10 µg salmon sperm DNA and 10 µg of linearized pPIC9::*phyA*. The
13 mixture is incubated for 1 h at 37°C, spread on *P. pastoris* minimal agar medium and
14 incubated for 2 to 5 d. Colonies growing on the minimal agar medium are streaked
15 for purity and analyzed for the presence of the integrated *phyA* by PCR and
16 Southern blot hybridization.

17 Expression of the *S. ruminantium* JY35 (ATCC 55785) phytase by
18 transformed *P. pastoris* cells is tested by growing the cells at 30°C under vigorous
19 aeration in a suitable liquid medium (e.g. buffered complex glycerol medium such as
20 BMGY) until a culture optical density (at 600 nm) (OD₆₀₀) of 2 to 6 is reached. The
21 cells are harvested and resuspended to an OD₆₀₀ of 1.0 in an inducing medium (e.g.,
22 buffered complex methanol medium, BMMY) and incubated for a further 3 to 5 days.
23 Cells and cell-free culture supernatant are collected and protein expression is
24 monitored by enzyme assay, SDS-PAGE, and western blot/immunodetection
25 techniques.

26
27 C. Cloning of the *Selenomonas ruminantium phyA* in a *Pichia pastoris* - specific
28 expression construct - A Further Example

29 An expression construct is constructed in which the region encoding the
30 mature PhyA is translationally fused with the secretion signal sequences found on
31 *P. pastoris* expression vectors (e.g., *Pichia* Expression Kit Instruction Manual,
32 Invitrogen Corporation, San Diego, CA) in order to express the *S. ruminantium*

1 phytase as a secreted product. The promoter and secretion signal sequences may
2 be replaced by those from other promoters that provide for efficient expression in
3 *Pichia*. The expression construct is introduced into *P. pastoris* cells by
4 transformation.

5 i. Construction of the *P. pastoris* expression vector

6 A number of *P. pastoris* expression vectors based on the *aox1* promoters and
7 α -Factor or *pho1* signal sequences are commercially available. In this example the
8 construct was prepared with pPICZ α A available from Invitrogen Corporation. The
9 region of *phyA* encoding the mature PhyA (i.e., the peptide secreted following
10 removal of the signal peptide) was amplified with oligonucleotide primers MATE (GC
11 GAA TTC GCC AAG GCG CCG GAG CAG AC SEQ ID NO. 6) and M13 Reverse.
12 The oligo MATE (SEQ ID NO. 6) was designed to contain an *EcoRI* restriction site
13 at its terminus to allow direct assembly of the amplified product with pPICZ α A. The
14 region of *phyA* amplified with MATE (SEQ ID NO. 6) and M13 Reverse was digested
15 with *EcoRI* and ligated into similarly cleaved pPICZ α A.

16 ii. Transformation of *P. pastoris*

17 The pPICZ α A::*phyA* ligation mix was used to transform competent *E. coli*
18 DH5 α cells. Transformed cells were spread on LB agar containing Zeocin (25
19 mg/mL) and incubated overnight at 37°C. Zeocin resistant colonies were screened
20 for the presence of the desired pPICZ α A::*phyA* construct by extracting pDNA and
21 subjecting the pDNA to agarose gel electrophoresis and restriction analysis. Positive
22 clones were further characterized by PCR and DNA sequence analysis. Plasmid
23 DNA was prepared from a 1 L culture of an *E. coli* clone carrying the desired
24 pPICZ α A::*phyA* construct. The pDNA is digested with *BglII* and analyzed by
25 agarose gel electrophoresis to confirm complete digestion of the vector. The
26 digested pDNA was extracted with phenol:chloroform, ethanol precipitated and
27 resuspended in sterile distilled H₂O to a final concentration of 1 μ g/ μ L.

28 In preparation for transformation, 50 mL of YPD broth were inoculated with *P.*
29 *pastoris* GS115 cells and incubated at 28°C and 250 RPM for 1 day. Subsequently,
30 5 mL of the 1 d culture was used to inoculate 50 mL of fresh YPD broth. The culture
31 was propagated overnight at 28°C and 250 RPM. The following morning, 5 mL of
32 this culture was used to inoculate 50 mL of fresh YPD broth. This culture was

1 incubated at 28°C and 250 RPM until the culture OD₆₀₀ reached approximately 1.2
2 (~ 6 h). The yeast cells from 20 ml of fresh culture were harvest by centrifugation,
3 washed once with and resuspended in 1 mL of room temperature 10 mM Tris, 1 mM
4 EDTA, 0.1 M LiCl, 0.1 M dithiothreitol buffer (pH 7.4). After a 1 h incubation at 30°C,
5 the cell suspension was washed once with 1 mL ice cold water and once with 1 mL
6 ice cold 1 M sorbitol. The cells were resuspended in 160 µL of ice cold 1 M sorbitol
7 (to obtain cell concentrations approaching 10¹⁰ cells/mL). Linearized
8 pPICZαA::phyA (5 to 10 µg) was mixed with 80 µL of cells, loaded into prechilled
9 electroporation cuvettes (0.2 cm inter-electrode distance) and incubated on ice for
10 5 min. A high voltage pulse (1.5 kV, 25 µF, 200 Ohms) was applied to the cuvette
11 with a Bio-Rad Gene Pulser™. Immediately following the pulse, 1 mL of ice cold 1M
12 sorbitol was added to the cuvette which was incubated subsequently for 2 h at 30°C.
13 The cell suspension was spread (100 to 200 µL per plate) on YPD agar medium
14 containing Zeocin (100 µg/mL) and incubated for 2 to 4 d at 30°C. Colonies growing
15 on the selective medium were streaked for purity and analyzed for the presence of
16 the integrated *phyA* by PCR and/or Southern blot hybridization.

17 iii. *Pichia pastoris* expression of the *S. ruminantium* JY35 phytase gene

18 Expression of the *S. ruminantium* JY35 phytase by transformed *P. pastoris*
19 cells was tested by growing transformed cells grown overnight in buffered complex
20 glycerol medium (e.g., buffered complex glycerol medium, BMGY, *Pichia* Expression
21 Kit Instruction Manual) at 28°C and 250 RPM and transferring them into inducing
22 medium (e.g., buffered complex methanol medium, BMMY). The cells harvested
23 from the BMGY medium were washed once with BMMY medium, resuspended in
24 BMMY to an OD₆₀₀ of 1.0 and incubated for a further 3 to 5 days at 28°C and 250
25 RPM. Methanol (0.005 volumes) was added every 24 h. Cells and cell free culture
26 supernatants were collected and assayed for phytase activity.

27 Sixteen *P. pastoris* pPICZαA::MATE transformants were tested for phytase
28 activity following 96 h growth in BMMY medium. The most active transformant,
29 named clone 17, was selected for further study. Growth and phytase production by
30 *P. pastoris* pPICZαA::MATE clone 17 and a negative clone (*P. pastoris* pPICZαA)
31 were monitored over a period of 9 d. Starter cultures were prepared by growing the
32 isolates overnight (28°C, 250 RPM) in 10 mL of BMGY (glycerol) medium. The cells

1 were harvested and duplicate cultures were prepared by resuspending the cells in
2 50 mL BMMY (methanol) medium to an approximate OD_{600} of 2.5. The resulting
3 cultures were transferred into 500 mL flasks and incubated at 28°C and 250 RPM.
4 Methanol was added every 24 h to a final concentration of 0.5%. Optical density and
5 phytase activity were measured over the time course of the experiment. The results
6 are presented in Table 4. Phytase activity was detected only in cultures carrying the
7 *S. ruminantium phyA* gene. These cultures produced up to 22.5 units of phytase
8 activity per mL after 210.5 h cultivation.

9 Phytase activity in shake flask cultures was increased through modification of
10 the induction protocol and medium composition. The phytase activity of clone 17 was
11 dramatically improved by increasing the initial cell density ($OD_{610} = 36.0$) of the
12 induced culture. After nearly 4 d growth (91.5 h), phytase activities greater than 40
13 and 20 units/mL were observed for whole culture and cell free supernatant samples,
14 respectively. The optical densities (OD_{610}) of these cultures were between 62 and
15 69. Experimental results suggest that the greater the culture biomass at the time
16 of methanol induction, the greater the yields of recombinant phytase. Biomass yields
17 as high as 150 g/L (dry weight) or optical densities of 1500 have been reported for
18 *Pichia* cultivated under optimal growth conditions in a tightly controlled fermentor
19 system operating with oxygen enrichment.

20 *Pichia* phytase yields were also increased by adding Tween-80 to the
21 medium. Surfactants have been shown previously to affect phytase production by
22 *Aspergillus carbonarius* (Al-Asheh and Duvnjak, 1994). The effect of incorporating
23 0, 0.02, 0.1 or 0.5 % Tween-80 on phytase yields of BMMY cultures of *P. pastoris*
24 pPICZ α A::MATE clone 17 is illustrated in Table 5. The cells from 2 d YPD cultures
25 were harvested and resuspended in BMMY ($OD_{610} = 8.3$). Triplicate flasks for each
26 concentration of Tween-80 were prepared and incubated at 28°C and 250 RPM.
27 Methanol (0.005 volumes) was added on a daily basis to the flasks. Phytase activity
28 increased more rapidly in cultures containing higher concentrations of Tween-80.
29 Furthermore, a larger proportion of the phytase activity was found in the supernatant
30 when higher Tween-80 concentrations were used. Phytase yields as high as 298
31 units/mL of shake flask culture have been achieved with a 9 d culture of clone 17
32 cultivated in BMMY medium amended with 0.5% Tween-80.

1 Cellular and supernatant proteins were analyzed by SDS-PAGE to confirm the
2 production of PhyA by *P. pastoris*. The presence of a 37 kDa protein band was
3 readily apparent when as little as 5 μ L of supernatant was resolved on a 12% SDS-
4 PAGE gel. The 37 kDa band was visible in the cellular protein sample but
5 represented less than 10% of that found in the corresponding amount of supernatant.
6 In addition to PhyA, supernatants from clone 17 contained very few additional
7 proteins (a useful characteristic of *Pichia* expression). The recombinant PhyA
8 protein comprised over 95% (estimated from SDS-PAGE gels) of the secreted
9 protein. The 37 kDa protein band was not present in the supernatant or cells of a
10 negative control culture (*P. pastoris* pPICZ α A).

11 Shake flask experiments with recombinant *P. pastoris* cells expressing the *S.*
12 *ruminantium* phytase (PhyA) have demonstrated the potential of this protein
13 production system. Significant gains in phytase yields will be obtained by cultivating
14 and inducing clone 17 in a fermentor. Additional gains in phytase yields may be
15 achieved by increasing gene copy number through further screening of independent
16 transformants or the use of multicopy vector systems. Spontaneous multiple plasmid
17 integration events occur in *Pichia* at a frequency between 1/10 and 1/100
18 transformants. It is not unrealistic to expect that a 10 fold gain in phytase yield (e.g.,
19 3,000 units/mL) may be readily achieved through manipulation of phytase gene copy
20 number and control of fermentation parameters. This would result in production
21 levels comparable to commercial *A. ficuum* phytase production systems. Yields for
22 these systems are believed to be around 3,000,000 units (μ mol Pi released/min) of
23 phytase activity per L of culture.

24 iv. The Activity of recombinant the *S. ruminantium* phytase (PhyA) on
25 grain substrates

26 The liberation of phosphate from corn by the recombinant *S. ruminantium*
27 JY35 phytase produced by *Pichia pastoris* was examined. Feed corn was ground
28 and sieved through a mesh to obtain a particle size between 1 - 3 mm. Ground corn
29 (0.5 g) was weighed into sterile 15 mL Falcon tubes to which 2 mL of 0.1 M sodium
30 acetate buffer (pH 5.0) was added. After addition of phytase, the reaction mixtures
31 were incubated at 37°C. Phosphate release was determined by measuring
32 supernatant phosphate. In order to measure the background phosphate, reaction

1 mixtures were prepared and terminated immediately through the addition of 5% (w/v)
2 TCA. All experiments were conducted in triplicate.

3 Incubation of corn in a sodium acetate buffer resulted in the release of
4 increasing amounts of phosphorus over time (Table 6). Although the addition of
5 phytase activity significantly increased the amount of phosphorus released, the rate
6 of phosphorus release decreased with time.

7 The concentration of phytase added to the incubation mixture also influenced
8 the amount of phosphorus released. Raising phytase concentrations from 0.08 units
9 to 0.48 units per g of corn resulted in increased levels of phosphorus in the
10 supernatant (Table 7). It should be noted that increasing the phytase concentration
11 from 0.32 to 0.48 units produced only a marginal increase in phosphorus released.

12
13 D. Cloning of the *Selenomonas ruminantium* phyA in a *Brassica napus* seed -
14 specific expression construct

15 Transformation and gene expression methods have been developed for a
16 wide variety of monocotyledonous and dicotyledonous crop species. In this example,
17 a *S. ruminantium* JY35 (ATCC 55785) phytase expression construct is constructed
18 in which the region encoding the mature PhyA is translationally fused with an oleosin
19 coding sequence in order to target seed oil body specific expression of the *S.*
20 *ruminantium* phytase. The promoter and/or secretion signal sequences may be
21 replaced by those from other promoters that provide for efficient expression in *B.*
22 *napus* or other transformable plant species. The expression construct is introduced
23 into *B. napus* cells by *Agrobacterium*-mediated transformation.

24 i. Construction of the *B. napus* expression vector

25 A number of expression vectors functional in *B. napus* are described in the
26 literature (Gelvin et al., 1993). In this example, the construct is prepared by replacing
27 the *E. coli* β -glucuronidase CDS of pCGOBPGUS (van Rooijen and Moloney, 1994)
28 with a fragment encoding the *phyA* mature CDS. This is accomplished by
29 subcloning the pCGOBPGUS *Pst*I *Kpn*I fragment, containing the oleosin
30 promoter::oleosin CDS:: β -glucuronidase CDS::NOS region, on to *Pst*I *Kpn*I- digested
31 pUCBM20 (Boehringer Mannheim Canada, Laval, PQ). This plasmid is called
32 pBMOBPGUS. The region of *phyA* encoding the mature PhyA is amplified with

1 oligonucleotide primers MATN (GA GGA TCC ATG GCC AAG GCG CCG GAG CAG
2 AC) (SEQ ID NO. 7) and M13 Reverse. The oligonucleotide MATN (SEQ ID NO.
3 7) was designed to contain a suitable restriction site at its terminus to allow direct
4 assembly of the amplified product with digested pBMOBPGUS. The *phyA* fragment
5 amplified with MATN (SEQ ID NO. 7) and M13 Reverse is digested with *NcoI* *SstI*
6 and ligated into similarly cleaved pBMOBPGUS to generate plasmid pBMOBP*phyA*.
7 The *B. napus* expression vector, pCGOBP*phyA*, is constructed by replacing the *PstI*
8 *KpnI* fragment from pCGOBPGUS with the *PstI* *KpnI* fragment from pBMOBP*phyA*,
9 containing the oleosin promoter::oleosin CDS::*phyA* CDS::NOS fragment.

10 ii. Transformation of *B. napus* and stable *PhyA* expression

11 Transgenic *B. napus* is prepared as described by van Rooijen and Moloney
12 (1994). *Agrobacterium tumefaciens* strain EHA101 is transformed by electroporation
13 with pCGOBP*phyA*. Cotyledonary petioles of *B. napus* are transformed with *A.*
14 *tumefaciens* EHA101 (pCGOBP*phyA*). Transgenic plants are regenerated from
15 explants that root on hormone-free MS medium containing 20 µg/mL kanamycin.
16 Young plants are assayed for NPTII activity, grown to maturity and allowed to self
17 pollenate and set seed. Seeds from individual transformants are pooled and part of
18 the seed sample is assayed for the presence of phytase activity and compared to
19 seeds from untransformed plants. Second generation plants (T2) are propagated
20 from the seeds of clones with the highest levels of phytase activity. Seeds from the
21 T2 plants homozygous for NPTII (hence also for *phyA*) are selected and used for
22 mass propagation of plants (T3) capable of producing the highest amounts of
23 phytase.

24
25 Example 8

26
27 Identification of Related Phytase Genes in Other Microorganisms

28 To identify a phytase gene related to *phyA*, hybridization analysis can be used
29 to screen nucleic acids from one or more ruminal isolates of interest using *phyA*
30 (SEQ ID NO. 1) or portions thereof as probes by known techniques (Sambrook,
31 1989; Ausubel, 1990) as described in example 4B. Related nucleic acids may be
32 cloned by employing known techniques. Radioisotopes (i.e., ³²P) may be required

1 when screening organisms with complex genomes in order to increase the sensitivity
2 of the analysis. Polymerase chain reaction (PCR) amplification may also be used to
3 identify genes related to *phyA*. Related sequences found in pure or mixed cultures
4 are preferentially amplified by PCR (and variations of such as Reverse Transcription
5 - PCR) with oligonucleotides primers designed using SEQ ID NO. 1. Amplified
6 products may be visualized by agarose gel electrophoresis and cloned using known
7 techniques. A variety of materials, including cells, colonies, plaques, and extracted
8 nucleic acids (e.g., DNA, RNA), may be examined by these techniques for the
9 presence of related sequences. Alternatively, known immunodetection techniques
10 employing antibodies specific to PhyA (SEQ ID NO. 2) can be used to screen whole
11 cells or extracted proteins of interest for the presence of related phytase(s).
12

Table 1. Phytase activity among rumen bacteria.

Phytase Activity	Microorganism	Number of isolates tested
Very Strong	<i>Prevotella</i> sp.	1
	<i>Selenomonas ruminantium</i>	11
Strong	<i>Prevotella ruminicola</i>	4
	<i>S. ruminantium</i>	13
Moderate	<i>Bacillus</i> sp.	1
	<i>Megasphaera elsdenii</i>	7
	<i>P. ruminicola</i>	6
	<i>S. ruminantium</i>	37
	<i>Treponema</i> sp.	1
Negative	<i>Anaerovibrio lipolytica</i>	2
	<i>Bacillus</i> sp.	4
	<i>Butyrivibrio fibrisolvens</i>	47
	<i>Clostridium</i> sp.	1
	<i>Coprococcus</i> sp.	3
	<i>Enterococcus</i> sp.	4
	<i>Eubacterium</i> sp.	7
	<i>Fibrobacter succinogenes</i>	8
	<i>Fusobacterium</i> sp.	3
	<i>Lachnospira multiparus</i>	4
	<i>Lactobacillus</i> sp.	20
	<i>M. elsdenii</i>	7
	<i>Peptostreptococcus</i> sp.	1
	<i>P. ruminicola</i>	41
	<i>Ruminobacter amylophilus</i>	4
	<i>Ruminococcus albus</i>	7
	<i>Ruminococcus flavefaciens</i>	10
	<i>S. ruminantium</i>	4
	<i>Streptococcus bovis</i>	48
	<i>Streptococcus milleri</i>	1
	<i>Staphylococcus</i> sp.	6
	<i>Succinovibrio dextrisolvens</i>	12
	<i>Treponema</i> sp.	12
	Unknown	8
Total isolates screened		345

Table 2. Phytase activity of selected rumen bacterial isolates.

Isolate	Phytase activity (mU*/mL)
<i>Selenomonas ruminantium</i> JY35	646
<i>Selenomonas ruminantium</i> KJ118	485
<i>Selenomonas ruminantium</i> BS131	460
<i>Selenomonas ruminantium</i> HD141	361
<i>Selenomonas ruminantium</i> HD86	286
<i>Selenomonas ruminantium</i> JY135	215
<i>Selenomonas ruminantium</i> D	69
<i>Selenomonas ruminantium</i> HD16	52
<i>Selenomonas ruminantium</i> BS114	47
<i>Selenomonas ruminantium</i> JY4	27
<i>Prevotella</i> sp. 46/5 ²	321
<i>Prevotella ruminicola</i> JY97	68
<i>Prevotella ruminicola</i> KJ182	61
<i>Prevotella ruminicola</i> JY106	49
<i>Megasphaera elsdenii</i> JY91	5

*U= μ moles, P_i released/min

Table 3. Overexpression of *S. ruminantium*¹ phytase in recombinant *E. coli* DH5 α .

Strain	Sample Composition	Units ² /mL	Specific Activity (Units/mg protein)
<i>E. coli</i> (pSrP.2)	cells supernatant	0.30 (0.08) ³ 0.308 (0.21)	1.56 (0.41) 2.64 (1.51)
<i>E. coli</i> (pSrPf6)	cells supernatant	0.91 (0.41) 5.10 (0.58)	6.42 (0.64) 22.83 (1.67)
<i>E. coli</i> (pSrP.2 <i>SphI</i>)	cells supernatant	ND ⁴ ND	ND ND

¹*S. ruminantium* JY35 is a crescent shaped-rod, an obligate anaerobe, produces propionic acid from the fermentation of glucose, ferments lactose, does not ferment glycerol, does not ferment mannitol (see also Bergey's Manual of Systematic Bacteriology, ed. John G. Holt, Williams and Wilkins, Baltimore, 1984)

²Units = μ moles P_i released/min

³Numbers in parentheses are standard errors

⁴ND = not detected

Table 4. Growth and phytase activity of *P. pastoris* cells transformed with pPICZ α A (negative control) or pPICZ α A::MATE (clone 17).

Culture	Time (h)	Optical Density (610 nm)	Phytase activity (μ mol/min/mL)	
			Culture	Supernatant
<i>P. pastoris</i> (pPICZ α A)	0.0	2.6	0.0	0.0
	20.5	10.1	0.0	0.0
	42.5	17.8	0.0	0.0
	68.0	17.0	0.0	0.0
	91.0	28.5	0.0	0.0
	138.5	39.3	0.0	0.0
	210.5	46.7	0.0	0.0
<i>P. pastoris</i> (pPICZ α A::MATE)	0.0	2.5	0.0	0.0
	20.5	11.3	1.9	0.1
	42.5	13.9	4.4	1.5
	68.0	12.9	8.0	2.7
	91.0	15.7	4.7	0.5
	138.5	18.3	12.6	5.3
	210.5	18.7	22.5	12.5

Table 5. The effect of Tween-80 concentration on growth and phytase activity of *P. pastoris* cells transformed with pPICZ α A::MATE (clone 17).

Time (d)	Sample (% Tween-80)	Optical Density (610 nm)	Phytase Activity (μ mol/min/mL)		Supernatant/ Culture Activity
			Culture	Supernatant	
2	0.0	24.3	4.1	2.2	0.55
	0.02	24.4	4.8	2.7	0.57
	0.1	25.1	5.2	3.2	0.61
	0.5	24.4	4.9	3.2	0.65
4	0.0	31.2	6.9	4.7	0.69
	0.02	31.0	8.2	5.5	0.67
	0.1	31.8	10.3	6.9	0.67
	0.5	29.2	10.3	9.1	0.88
8	0.0	32.8	10.6	5.9	0.55
	0.02	30.4	14.8	9.8	0.67
	0.1	33.9	20.2	17.2	0.86
	0.5	33.8	22.1	18.9	0.86

Table 6. The effect of incubation period and recombinant *S. ruminantium* JY35 phytase (2 units/g of corn) on phosphate release from corn.

Sample	Length of incubation (h)	Phosphate concentration (μmoles/mL)
No phytase	1	0.85
	2	1.72
	3	2.56
	4	3.77
	5	4.35
Phytase	1	4.76
	2	6.83
	3	7.72
	4	8.41
	5	8.49

Table 7. The effect of recombinant *S. ruminantium* JY35 phytase concentration on phosphate release from corn.

Phytase activity (units/g of corn)	Phosphate concentration (μmoles/g of corn)
0.08	11.8
0.16	14.8
0.24	22.5
0.32	23.0
0.40	23.2
0.48	23.8
0.56	23.8
0.64	23.6
0.72	23.8

REFERENCES

- Al-Asheh, S. And Z. Duvnjak. 1994. The effect of surfactants on the phytase production and the reduction of the phytic content in canola meal by *Aspergillus carbonarius* during a solid state fermentation process. *Biotechnol. Lett.* 16:183-188.
- Ausubel, F.A., R. Brent, R.E. Kingston, D.D. Moore, J.G. Sneiderman, J.A. Smith and K. Struhl. (eds.) 1990. Current protocols in molecular biology. Green Publishing and Wiley-Interscience, New York.
- Brosius, J., M. Erfle and J. Storella. 1985. Spacing of the -10 and -35 regions in the *tac* promoter. *J. Biol. Chem.* 260:3539-3541.
- Bryant, M.P. and L.A. Burkey. 1953. Cultural methods and some characteristics of some of the numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* 36:205-217.
- Cheng, E.W., G. Hall and W. Burroughs. 1955. A method for the study of cellulose digestion by washed suspensions of rumen microorganisms. *J. Dairy Sci.* 38:1255-1230.
- Cheng, K.-J. and J.W. Costerton, 1973. Localization of alkaline phosphatase in three Gram-negative rumen bacteria. *J. Bacteriol.* 116:424-440.
- Dayhoff, M.O., R.M. Schwartz and B.C. Orcutt. 1978. A model of evolutionary change in proteins. In: Atlas of Protein Sequence and Structure. Volume 5, Supplement 3, Chapter 22, pp 345-352.
- Ellis, S.B., P.F. Brust, P.J. Koutz, A.F. Waters, M.M. Harpold and R.R. Gingeras. 1985. Isolation of alcohol oxidase and two other methanol regulated genes from the yeast, *Pichia pastoris*. *Mol. Cell. Biol.* 5:1111-1121.

- 1 Fiske, C.H. and Y. Subbarow. 1925. The colorimetric determination of phosphorus.
2 J. Biol. Chem. 66:376-400.
3
- 4 Gelvin, S.B., R.A. Schilperoort and D.P.S. Verma (eds.). 1993. Plant Molecular
5 Biology Manual. Kluwer Academic Publishers, Boston, MA.
6
- 7 Graf, E. (ed.). 1986. Phytic acid, chemistry and applications. Pilatus Press.
8 Minneapolis, MN. 344 pp.
9
- 10 Howson, S.J. and R.P. Davis. 1983. Production of phytate-hydrolysing enzyme by
11 some fungi. *Enzyme Microb. Technol.* 5:377-382.
12
- 13 Hu, Y.J., D.C. Smith, K.-J. Cheng and C.W. Forsberg. 1991. Cloning of a xylanase
14 gene from *Fibrobacter succinogenes* 135 and its expression in *Escherichia*
15 *coli*. *Can.J. Microbiol.* 37:554-561.
16
- 17 Hungate, R.E. 1950. The anaerobic mesophilic cellulolytic bacteria. *Bacteriol. Rev.*
18 14:1-49.
19
- 20 Laemmli, U.K. 1970. Cleavage of the structural proteins during assembly of the
21 head of bacteriophage T4. *Nature* 227:680-685.
22
- 23 Priefer, U., R. Simon and A. Puhler. 1984. Cloning with cosmids. *In*: Puhler, A. and
24 K.N. Timmis (eds) Advanced molecular genetics. Springer-Verlag, New York.
25 pp.190-201.
26
- 27 Raun, A., E. Cheng and W. Burroughs. 1956. Phytate phosphorus hydrolysis and
28 availability to rumen microorganisms. *Agric. Food Chem.* 4:869-871.
29
- 30 Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular cloning. A laboratory
31 manual. 2nd. edn. Cold Spring Harbor Laboratory Press. Cold Spring Harbor,
32 NY.

1 Scott, H.W. and B.A. Dehority. 1965. Vitamin requirements of several cellulolytic
2 bacteria. J. Bacteriol. 89:1169-1175.

3
4 Shieh, T.R. and J.H. Ware. 1968. Survey of microorganisms for the production of
5 extracellular phytase. Appl. Microbiol. 16:1348-1351.

6
7 van Gorcom, R.F.M. and C.A.M.J Van Den Hondel. 1993. Cloning, characterization
8 and overexpression of the phytase gene (*phyA*) of *Aspergillus niger*. Gene
9 127:87-94.

10
11 van Hartingsveldt W., C.M.J. van Zeijl, M.G. Hartevelde, R.J. Gouka, M.E.G.
12 Suykerbuyk, R.G.M. Luiten, P.A. Van Paridon, G.C.M. Selten, A.E. Veenstra,
13 van Rooijen, G.J.H. and M. M. Moloney. 1994. Plant seed oil-bodies as
14 carriers for foreign proteins. Bio/Technology 13:72-77.

15
16 von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites.
17 Nucleic Acids Res. 14:4683-4690.

18
19 Wong, S.-L. 1989. Development of an inducible and enhancible expression and
20 secretion system in *Bacillus subtilis*. Gene 83:215-223.

21
22 All publications mentioned in this specification are indicative of the level of skill
23 of those skilled in the art to which this invention pertains. All publications are herein
24 incorporated by reference to the same extent as if each individual publication was
25 specifically indicated to be incorporated by reference.

26 Although the foregoing invention has been described in some detail by way
27 of illustration and example for purposes of clarity and understanding, it will be
28 obvious that certain changes and modifications may be practised within the scope
29 of the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Cheng, Kuo-Joan
Selinger, Leonard B.
Yanke, Lindsey J.
Bae, Hee-Dong
Zhou, Lu Ming
Forsberg, Cecil W.

(ii) TITLE OF INVENTION: DNA sequences encoding phytases of
ruminal microorganisms.

(iii) NUMBER OF SEQUENCES: 7

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: May 23, 1997
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Mary Jane McKay-Carey
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(C) REFERENCE/DOCKET NUMBER: 37003W00

(ix) TELECOMMUNICATION INFORMATION:

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(B) TELEFAX: (403) 421-0834

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1401 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Selenomonas ruminantium*

(B) STRAIN: JY35

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: Genomic DNA library

(B) CLONE: pSrP.2

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 231..1268

(C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION: /codon_start= 231

/function= "Dephosphorylation of phytic acid"

/product= "Phytase"

/evidence= EXPERIMENTAL

/gene= "phyA"

/number= 1

/standard_name= "myo-inositol hexaphosphate
phosphohydrolase"

/citation= ([1])

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 231..311

(C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION: /codon_start= 1

/function= "phytase secretion"

/product= "Signal peptide"

/evidence= EXPERIMENTAL

/citation= ([1])

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 312..1268

(C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION: /codon_start= 312

/product= "Phytase"

/evidence= EXPERIMENTAL

/number= 2

/citation= ([1])

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGTCCACGGA GTCACCCTAC TATACGACGT ATGTGAAGTT CACGTCGAAG TTCTAGGGAA	60
TCACCGATTC GTGCAGGATT TTACCACTTC CTGTTGAAGC GGATGAGAAG GGGAACCGCG	120
AAGCGGTGGA AGAGGTGCTG CACGACGGAC GATCGCGCTG AATGAATCAG TGCTTCCTAA	180

CTATTGGGAT TCCGCGCAGA CGCGCGGATG GAGTAAAGGA GTAAGTTGTT ATG AAA	236
Met Lys	
-27	
TAC TGG CAG AAG CAT GCC GTT CTT TGT AGT CTC TTG GTC GGC GCA TCC	284
Tyr Trp Gln Lys His Ala Val Leu Cys Ser Leu Leu Val Gly Ala Ser	
-25 -20 -15 -10	
CTC TGG ATA CTG CCG CAG GCC GAT GCG GCC AAG GCG CCG GAG CAG ACG	332
Leu Trp Ile Leu Pro Gln Ala Asp Ala Ala Lys Ala Pro Glu Gln Thr	
-5 1 5	
GTG ACG GAG CCC GTT GGG AGC TAC GCG CGC GCG GAG CGG CCG CAG GAC	380
Val Thr Glu Pro Val Gly Ser Tyr Ala Arg Ala Glu Arg Pro Gln Asp	
10 15 20	
TTC GAG GGC TTT GTC TGG CGC CTC GAC AAC GAC GGC AAG GAG GCG TTG	428
Phe Glu Gly Phe Val Trp Arg Leu Asp Asn Asp Gly Lys Glu Ala Leu	
25 30 35	
CCG CGT AAT TTC CGC ACG TCG GCT GAC GCG CTG CGC GCG CCG GAG AAG	476
Pro Arg Asn Phe Arg Thr Ser Ala Asp Ala Leu Arg Ala Pro Glu Lys	
40 45 50 55	
AAA TTC CAT CTC GAC GCC GCG TAT GTA CCG TCG CGC GAG GGC ATG GAT	524
Lys Phe His Leu Asp Ala Ala Tyr Val Pro Ser Arg Glu Gly Met Asp	
60 65 70	
GCA CTC CAT ATC TCG GGC AGT TCC GCA TTC ACG CCG GCG CAG CTC AAG	572
Ala Leu His Ile Ser Gly Ser Ser Ala Phe Thr Pro Ala Gln Leu Lys	
75 80 85	
AAC GTT GCC GCG AAG CTG CGG GAG AAG ACG GCT GGC CCC ATC TAC GAT	620
Asn Val Ala Ala Lys Leu Arg Glu Lys Thr Ala Gly Pro Ile Tyr Asp	
90 95 100	
GTC GAC CTA CGG CAG GAG TCG CAC GGC TAT CTC GAC GGT ATC CCC GTG	668
Val Asp Leu Arg Gln Glu Ser His Gly Tyr Leu Asp Gly Ile Pro Val	
105 110 115	
AGC TGG TAC GGC GAG CGC GAC TGG GCA AAT CTC GGC AAG AGC CAG CAT	716
Ser Trp Tyr Gly Glu Arg Asp Trp Ala Asn Leu Gly Lys Ser Gln His	
120 125 130 135	
GAG GCG CTC GCC GAC GAG CGG CAC CGC TTG CAC GCA GCG CTC CAT AAG	764
Glu Ala Leu Ala Asp Glu Arg His Arg Leu His Ala Ala Leu His Lys	
140 145 150	
ACG GTC TAC ATC GCG CCG CTC GGC AAG CAC AAG CTC CCC GAG GGC GGC	812
Thr Val Tyr Ile Ala Pro Leu Gly Lys His Lys Leu Pro Glu Gly Gly	
155 160 165	
GAA GTC CGC CGC GTA CAG AAG GTG CAG ACG GAA CAG GAA GTC GCC GAG	860
Glu Val Arg Arg Val Gln Lys Val Gln Thr Glu Gln Glu Val Ala Glu	
170 175 180	

GCC GCG GGG ATG CGC TAT TTC CGC ATC GCG GCG ACG GAT CAT GTC TGG 908
 Ala Ala Gly Met Arg Tyr Phe Arg Ile Ala Ala Thr Asp His Val Trp
 185 190 195

CCA ACG CCG GAG AAC ATC GAC CGC TTC CTC GCG TTT TAC CGC ACG CTG 956
 Pro Thr Pro Glu Asn Ile Asp Arg Phe Leu Ala Phe Tyr Arg Thr Leu
 200 205 210 215

CCG CAG GAT GCG TGG CTC CAT TTC CAT TGT GAA GCC GGT GTC GGC CGC 1004
 Pro Gln Asp Ala Trp Leu His Phe His Cys Glu Ala Gly Val Gly Arg
 220 225 230

ACG ACG GCG TTC ATG GTC ATG ACG GAT ATG CTG AAG AAC CCG TCC GTA 1052
 Thr Thr Ala Phe Met Val Met Thr Asp Met Leu Lys Asn Pro Ser Val
 235 240 245

TCG CTC AAG GAC ATC CTC TAT CGC CAG CAC GAG ATC GGC GGC TTT TAC 1100
 Ser Leu Lys Asp Ile Leu Tyr Arg Gln His Glu Ile Gly Gly Phe Tyr
 250 255 260

TAC GGG GAG TTC CCC ATC AAG ACG AAG GAT AAA GAT AGC TGG AAG ACG 1148
 Tyr Gly Glu Phe Pro Ile Lys Thr Lys Asp Lys Asp Ser Trp Lys Thr
 265 270 275

AAA TAT TAT AGG GAA AAG ATC GTG ATG ATC GAG CAG TTC TAC CGC TAT 1196
 Lys Tyr Tyr Arg Glu Lys Ile Val Met Ile Glu Gln Phe Tyr Arg Tyr
 280 285 290 295

GTG CAG GAG AAC CGC GCG GAT GGC TAC CAG ACG CCG TGG TCG GTC TGG 1244
 Val Gln Glu Asn Arg Ala Asp Gly Tyr Gln Thr Pro Trp Ser Val Trp
 300 305 310

CTC AAG AGC CAT CCG GCG AAG GCG TAAAGCGCA GCGGCGGCT CGGAGTCAGG 1298
 Leu Lys Ser His Pro Ala Lys Ala
 315

GAAATGGCGC TGCCAGCACG GGACGCGCGG CGGCGGATGC TCGCCCGGTC AGGGATGATT 1358

GACGACAGCC AGAGAAGAAA GGATGGTTTT ATGAGGTGGA TCC 1401

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 346 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Tyr Trp Gln Lys His Ala Val Leu Cys Ser Leu Leu Val Gly
 -27 -25 -20 -15

Ala Ser Leu Trp Ile Leu Pro Gln Ala Asp Ala Ala Lys Ala Pro Glu
 -10 -5 1 5
 Gln Thr Val Thr Glu Pro Val Gly Ser Tyr Ala Arg Ala Glu Arg Pro
 10 15 20
 Gln Asp Phe Glu Gly Phe Val Trp Arg Leu Asp Asn Asp Gly Lys Glu
 25 30 35
 Ala Leu Pro Arg Asn Phe Arg Thr Ser Ala Asp Ala Leu Arg Ala Pro
 40 45 50
 Glu Lys Lys Phe His Leu Asp Ala Ala Tyr Val Pro Ser Arg Glu Gly
 55 60 65
 Met Asp Ala Leu His Ile Ser Gly Ser Ser Ala Phe Thr Pro Ala Gln
 70 75 80 85
 Leu Lys Asn Val Ala Ala Lys Leu Arg Glu Lys Thr Ala Gly Pro Ile
 90 95 100
 Tyr Asp Val Asp Leu Arg Gln Glu Ser His Gly Tyr Leu Asp Gly Ile
 105 110 115
 Pro Val Ser Trp Tyr Gly Glu Arg Asp Trp Ala Asn Leu Gly Lys Ser
 120 125 130
 Gln His Glu Ala Leu Ala Asp Glu Arg His Arg Leu His Ala Ala Leu
 135 140 145
 His Lys Thr Val Tyr Ile Ala Pro Leu Gly Lys His Lys Leu Pro Glu
 150 155 160 165
 Gly Gly Glu Val Arg Arg Val Gln Lys Val Gln Thr Glu Gln Glu Val
 170 175 180
 Ala Glu Ala Ala Gly Met Arg Tyr Phe Arg Ile Ala Ala Thr Asp His
 185 190 195
 Val Trp Pro Thr Pro Glu Asn Ile Asp Arg Phe Leu Ala Phe Tyr Arg
 200 205 210
 Thr Leu Pro Gln Asp Ala Trp Leu His Phe His Cys Glu Ala Gly Val
 215 220 225
 Gly Arg Thr Thr Ala Phe Met Val Met Thr Asp Met Leu Lys Asn Pro
 230 235 240 245
 Ser Val Ser Leu Lys Asp Ile Leu Tyr Arg Gln His Glu Ile Gly Gly
 250 255 260
 Phe Tyr Tyr Gly Glu Phe Pro Ile Lys Thr Lys Asp Lys Asp Ser Trp
 265 270 275

Lys Thr Lys Tyr Tyr Arg Glu Lys Ile Val Met Ile Glu Gln Phe Tyr
 280 285 290

Arg Tyr Val Gln Glu Asn Arg Ala Asp Gly Tyr Gln Thr Pro Trp Ser
 295 300 305

Val Trp Leu Lys Ser His Pro Ala Lys Ala
 310 315

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide SrPr6"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Selenomonas ruminantium
- (B) STRAIN: JY35

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Genomic DNA library
- (B) CLONE: pSrP.2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGGATGCTT CTGCCAGTAT

20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide SrPf6"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Selenomonas ruminantium

(B) STRAIN: JY35

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Genomic DNA library
- (B) CLONE: pSrP.2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGTCCACGGA GTCACCCTAC

20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide MATE2"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Selenomonas ruminantium
- (B) STRAIN: JY35

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Genomic DNA library
- (B) CLONE: pSrP.2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGAATTCAT GGCCAAGGCG CCGGAGCAGA C

31

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide MATE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Selenomonas ruminantium
- (B) STRAIN: JY35

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Genomic DNA library
- (B) CLONE: pSrP.2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGAATTCGC CAAGGCGCCG GAGCAGAC

28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide MATN"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Selenomonas ruminantium
- (B) STRAIN: JY35

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Genomic DNA library
- (B) CLONE: pSrP.2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAGGATCCAT GGCCAAGGCG CCGGAGCAGA C

31

1 WE CLAIM:

- 2 1. A purified and isolated DNA encoding a phytase of a ruminal microorganism.
- 3
- 4 2. A purified and isolated DNA according to claim 1 wherein said ruminal
- 5 microorganism is a prokaryote.
- 6
- 7 3. A purified and isolated DNA according to claim 1 wherein said ruminal
- 8 microorganism is of the genus *Selenomonas*, *Prevotella*, *Treponema* or
- 9 *Megasphaera*.
- 10
- 11 4. A purified and isolated DNA according to claim 1 wherein said ruminal
- 12 microorganism is *Selenomonas ruminantium*, *Prevotella ruminicola*,
- 13 *Treponema bryantii* or *Megasphaera elsdenii*.
- 14
- 15 5. A purified and isolated DNA according to claim 1 wherein said ruminal
- 16 microorganism is *Selenomonas ruminantium*.
- 17
- 18 6. A purified and isolated DNA according to claim 1 wherein said ruminal
- 19 microorganism is *Selenomonas ruminantium* JY35 (ATCC 55785).
- 20
- 21 7. A purified and isolated DNA according to claim 1, said DNA being capable of
- 22 hybridizing under stringent conditions with a probe comprising at least 25
- 23 continuous nucleotides of nucleotide sequence SEQ ID NO. 1.
- 24
- 25 8. A purified and isolated DNA according to claim 1, said phytase comprising
- 26 amino acid sequence SEQ ID NO. 2.
- 27
- 28 9. A purified and isolated DNA according to claim 1, said DNA comprising
- 29 nucleotide sequence SEQ ID NO. 1.
- 30
- 31 10. A purified and isolated DNA according to claim 1, said DNA comprising
- 32 nucleotides 312-1268 of SEQ ID NO. 1.

- 1 11. A purified and isolated DNA according to claim 1, wherein said encoded
2 phytase has the following characteristics:
3 a) a molecular mass of about 37 kDa;
4 b) is active within a pH range of about 3.0 to 6.0; and
5 c) is active within a temperature range of about 4 to 55°C.
6
- 7 12. A purified and isolated DNA according to claim 11 wherein said encoded
8 phytase is active within a temperature range of about 20 to 55°C.
9
- 10 13. A purified and isolated DNA according to claim 11 wherein said encoded
11 phytase is active within a temperature range of about 35 to 40°C.
12
- 13 14. A purified and isolated DNA according to claim 11, wherein the encoded
14 phytase has the following additional characteristic:
15 d) a specific activity at least two fold higher than that of *Aspergillus ficuum*
16 NRRI 3135 PhyA as measured by the release of inorganic phosphate.
17
- 18 15. An expression construct capable of directing the expression of a phytase in
19 a suitable host cell, said expression construct comprising a DNA encoding a
20 phytase of a ruminal microorganism operably linked to control sequences
21 compatible with said host cell.
22
- 23 16. An expression construct according to claim 15 wherein said ruminal
24 microorganism is *Selenomonas ruminantium*.
25
- 26 17. An expression construct according to claim 15 wherein said encoded phytase
27 comprises amino acid sequence SEQ ID NO. 2.
28
- 29 18. A host cell transformed with a DNA encoding a phytase of a ruminal
30 microorganism so that the host cell can express the phytase encoded by said
31 DNA.
32

- 1 19. A transformed host cell according to claim 18 wherein said ruminal
2 microorganism is *Selenomonas ruminantium*.
3
- 4 20. A transformed host cell according to claim 18 wherein said encoded phytase
5 comprises amino acid sequence SEQ ID NO. 2.
6
- 7 21. A transformed host cell according to claim 18 wherein said host cell is a
8 eukaryote.
9
- 10 22. A transformed host cell according to claim 18 wherein said host cell is a
11 prokaryote.
12
- 13 23. A transformed host cell according to claim 18 wherein said host cell is a
14 *Pichea pastoris* cell.
15
- 16 24. A transformed host cell according to claim 18 wherein said host cell is a
17 *Bacillus subtilis* cell..
18
- 19 25. A transformed host cell according to claim 18 wherein said host cell is an *E.*
20 *coli* cell
21
- 22 26. *Selenomonas ruminantium* JY35 (ATCC 55785).
23
- 24 27. A transgenic plant transformed with a DNA encoding a phytase of a ruminal
25 microorganism so that the phytase encoded by said DNA can be expressed
26 by said plant.
27
- 28 28. A transgenic plant according to claim 27 wherein said ruminal microorganism
29 is *Selenomonas ruminantium*.
30
- 31 29. A transgenic plant according to claim 27 wherein said encoded phytase
32 comprises amino acid sequence SEQ ID NO. 2.

- 1 30. A phytase of a ruminal microorganism.
- 2
- 3 31. A phytase according to claim 30 wherein said ruminal microorganism is
- 4 *Selenomonas ruminantium*.
- 5
- 6 32. A phytase according to claim 30 wherein said phytase has the following
- 7 characteristics:
- 8 a) a molecular mass of about 37 kDa;
- 9 b) is active within a pH range of about 3.0 to 6.0; and
- 10 c) is active within a temperature range of about 4 to 55°C.
- 11
- 12 33. A phytase according to claim 32 having the following additional characteristic:
- 13 d) a specific activity at least two fold higher than that of *Aspergillus ficuum*
- 14 NRRI 3135 PhyA as measured by the release of inorganic phosphate.
- 15
- 16 34. A phytase according to claim 30, comprising a contiguous amino acid
- 17 sequence residing within amino acid sequence SEQ ID NO. 2.
- 18
- 19 35. A phytase according to claim 30 comprising amino acid sequence SEQ ID
- 20 NO. 2.
- 21
- 22 36. A feed composition comprising a feedstuff treated with a phytase of a ruminal
- 23 microorganism.
- 24
- 25 37. A feed composition according to claim 36 wherein said ruminal microorganism
- 26 is *Selenomonas ruminantium*.
- 27
- 28 38. A feed composition according to claim 36 wherein said phytase comprises
- 29 amino acid sequence SEQ ID NO. 2.
- 30

- 1 39. A feed composition according to claim 36 containing a sufficient amount of
2 said phytase to provide up to about 2000 Units (μ moles phosphate
3 released/minute) of phytase activity per kg feed composition.
4
- 5 40. A feed composition according to claim 36 containing a sufficient amount of
6 said phytase to provide up to about 1000 Units of phytase activity per kg feed
7 composition.
8
- 9 41. A feed composition according to claim 36 containing a sufficient amount of
10 said phytase to provide from about 50 to 800 Units of phytase activity per kg
11 feed composition.
12
- 13 42. A feed composition according to claim 36 containing a sufficient amount of
14 said phytase to provide from about 300 to 800 Units of phytase activity per kg
15 feed composition.
16
- 17 43. A feed additive comprising a preparation of lyophilized microorganisms, said
18 microorganisms expressing a phytase of a ruminal microorganism under
19 normal growing conditions.
20
- 21 44. A feed additive according to claim 43 wherein said microorganism is
22 *Selenomonas ruminantium*.
23
- 24 45. A feed additive according to claim 43 wherein said microorganism is a
25 recombinant microorganism transformed with a DNA encoding said phytase
26 of said ruminal microorganism.
27
- 28 46. A feed additive according to claim 45 wherein said ruminal microorganism is
29 *Selenomonas ruminantium*.
30
- 31 47. A feed additive according to claim 45 wherein said expressed phytase
32 comprises amino acid sequence SEQ ID NO. 2.

- 1 48. A feed additive for treatment of a feedstuff, said feed additive comprising a
2 phytase of a ruminal microorganism.
3
- 4 49. A feed additive according to claim 48 wherein said microorganism is
5 *Selenomonas ruminantium*.
6
- 7 50. A feed additive according to claim 48 wherein said phytase comprises amino
8 acid sequence SEQ ID NO. 2.
9
- 10 51. A method for producing a phytase, comprising:
11 (a) transforming at least one host cell with a DNA encoding a phytase of
12 a ruminal microorganism so that said host cell can express said phytase; and
13 (b) growing a culture of said host cells under conditions conducive to the
14 expression of said phytase by said host cells.
15
- 16 52. A method according to claim 51 comprising the further step of:
17 (c) extracting said phytase from said culture.
18
- 19 53. A method according to claim 51 wherein said ruminal microorganism is
20 *Selenomonas ruminantium*.
21
- 22 54. A method according to claim 51 wherein said phytase comprises amino acid
23 sequence SEQ ID NO. 2.
24
- 25 55. A method for producing a transgenic plant, comprising:
26 (a) transforming a plant with a DNA encoding a phytase of a ruminal
27 microorganism so that said plant can express said phytase; and
28 (b) growing said plant under conditions conducive to the expression of said
29 phytase by said plant.
30
- 31 56. A method according to claim 55 wherein said ruminal microorganism is
32 *Selenomonas ruminantium*.

- 1 57. A method according to claim 55 wherein said phytase comprises amino acid
2 sequence SEQ ID NO. 2:
3
- 4 58. A method for improving dietary phytate utilization by an animal, comprising
5 feeding said animal a diet which includes an effective amount of a phytase of
6 a ruminal microorganism.
7
- 8 59. A method according to claim 58 wherein said ruminal microorganism is
9 *Selenomonas ruminantium*.
10
- 11 60. A method according to claim 58 wherein said phytase comprises amino acid
12 sequence SEQ ID NO. 2.
13
- 14 61. A method according to claim 58 wherein said diet includes drinking water and
15 said phytase is included in said drinking water.
16
- 17 62. A method according to claim 58 wherein said phytase is provided in a mineral
18 block for consumption by said animal.
19
- 20 63. A method according to claim 58 wherein said phytase is provided in a pill for
21 consumption by said animal.
22
- 23 64. A method according to claim 58 wherein said phytase is provided in a gel
24 formulation for consumption by said animal.
25
- 26 65. A method according to claim 58 wherein said phytase is sprayed in a liquid
27 formulation onto a feedstuff for consumption by said animal.
28
- 29 66. A method according to claim 58 wherein said phytase is provided in a
30 pelletized feedstuff for consumption by said animal.
31

- 1 67. A method according to claim 58 wherein a feedstuff for consumption by said
2 animal is treated with a preparation of lyophilized microorganisms, said
3 microorganisms expressing said phytase under normal growing conditions.
4
- 5 68. A method for assaying phytase activity of a microorganism, comprising the
6 steps of:
7 (a) providing a growth medium upon which colonies of microorganisms
8 have been grown, said medium containing a source of phytate;
9 (b) contacting said medium with an aqueous solution of cobalt chloride;
10 and
11 (c) examining said medium for zones of clearing,
12
13 whereby false positive results caused by microbial acid production are
14 eliminated.
15
- 16 69. A method according to claim 68 wherein after step (b), said medium is
17 contacted with an aqueous solution of ammonium molybdate and an
18 aqueous solution of ammonium vanadate;
19
- 20 70. A method according to claim 68 wherein said medium is contacted with said
21 aqueous solution of cobalt chloride for at least about 5 minutes.
22
- 23 71. A method according to claim 68 wherein said medium is contacted with said
24 aqueous solutions of ammonium molybdate and ammonium vanadate for at
25 least about 5 minutes.
26
- 27 72. A method according to claim 68 wherein said medium is contacted with said
28 aqueous solutions of ammonium molybdate and ammonium vanadate
29 simultaneously.
30
- 31 73. A method according to claim 68 wherein the concentration of said aqueous
32 solution of cobalt chloride is about 2% (weight/volume).

- 1 74. A method according to claim 69 wherein the concentration of said aqueous
2 solution of ammonium molybdate is about 6% (weight/volume) and the
3 concentration of said aqueous solution of ammonium vanadate is about 0.5%
4 (weight/volume).
5
- 6 75. A method for identifying a nucleic acid molecule from an organism, said
7 nucleic acid molecule encoding a phytase, said method comprising the steps
8 of:
9
- 10 (a) isolating nucleic acid molecules from said organism;
11
- 12 (b) performing nucleic acid hybridization under conditions of moderate to
13 high stringency with said nucleic acid molecules and a labelled
14 hybridization probe having a nucleotide sequence comprising at least
15 25 continuous nucleotides of SEQ ID NO: 1.
16
- 17 76. A method according to claim 75 wherein said hybridization conditions are of
18 moderate stringency.
19
- 20 77. A method according to claim 75 wherein said hybridization conditions are of
21 high stringency.

1/10

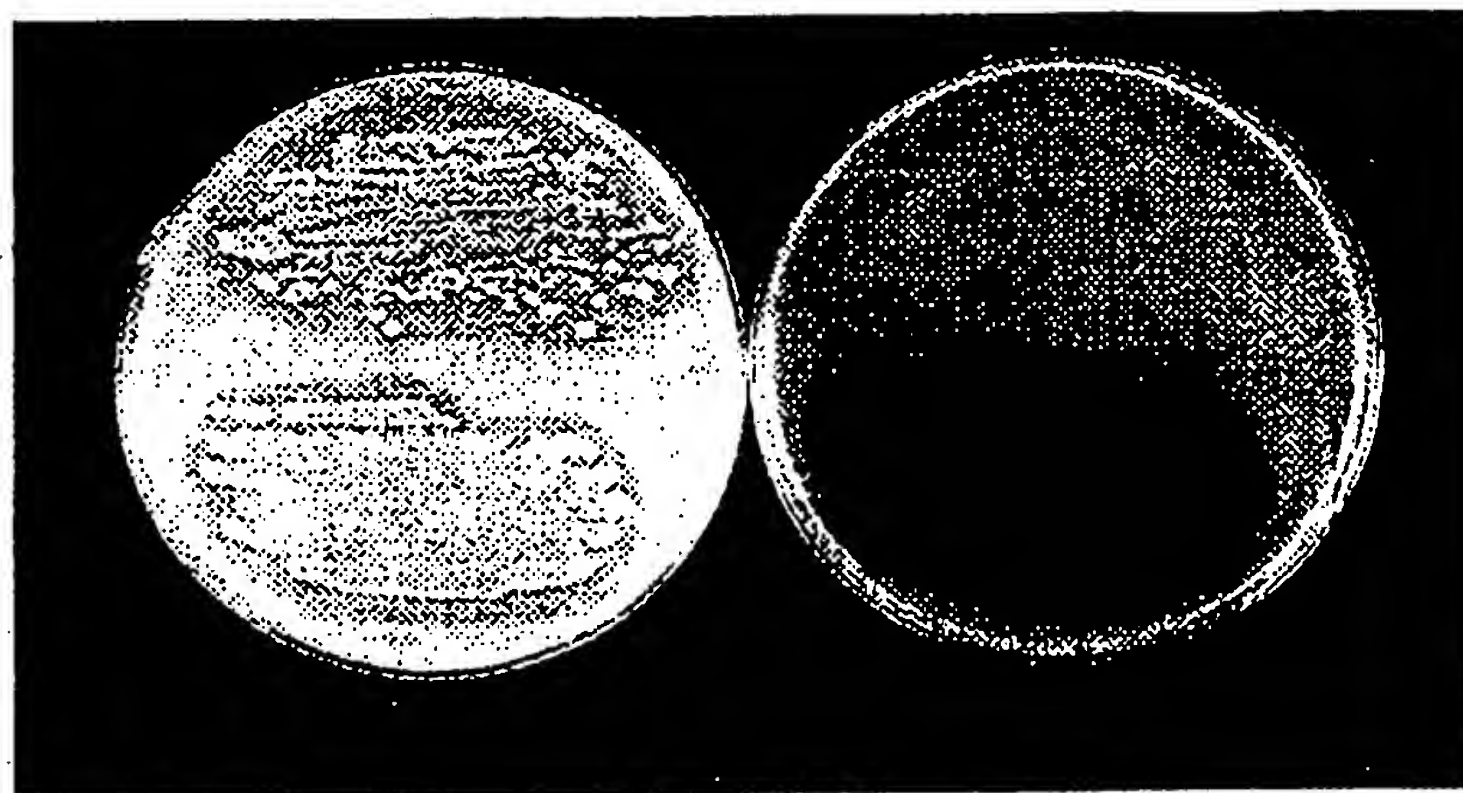


Figure 1

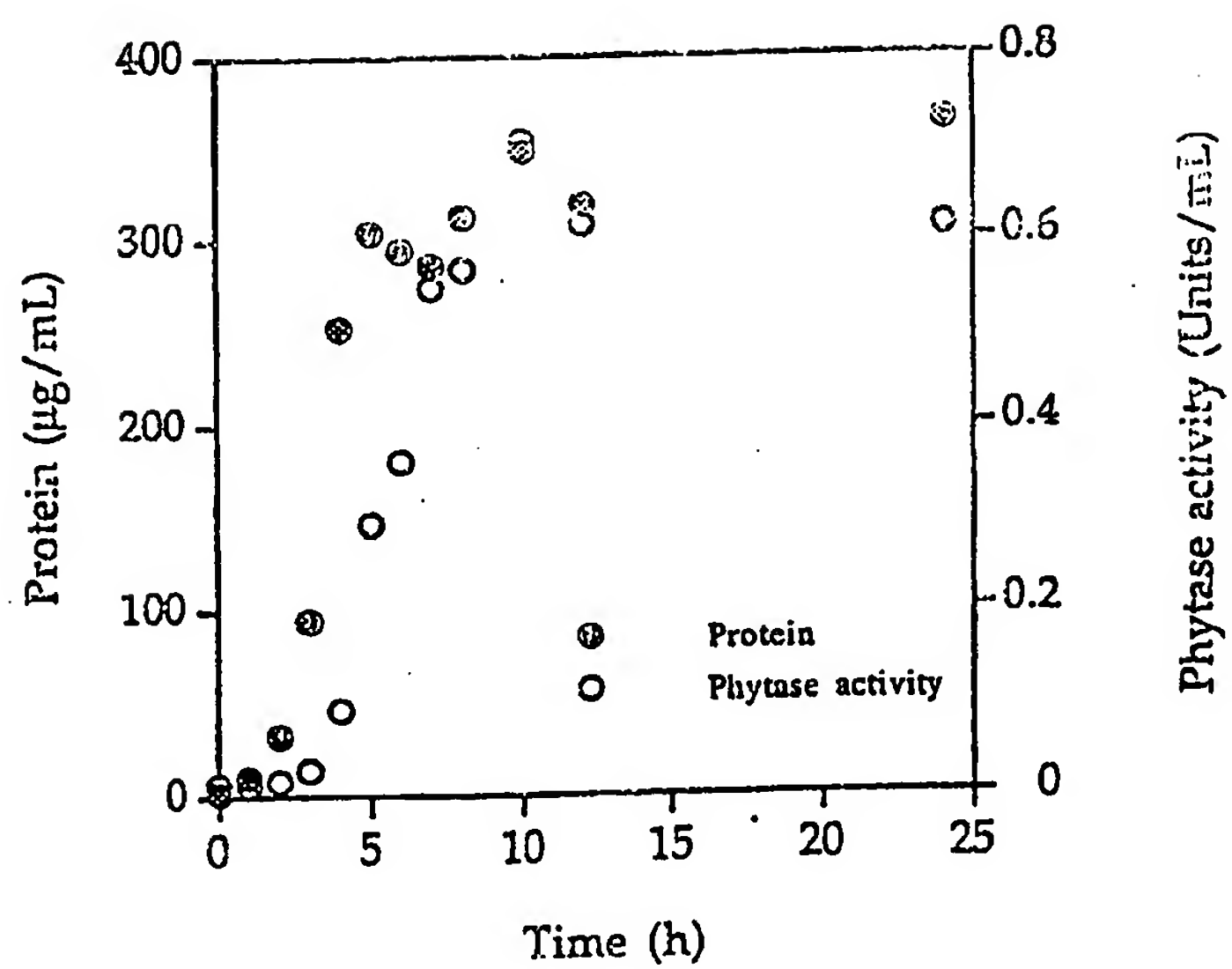


Figure 2

Fig.3A



Fig. 3D

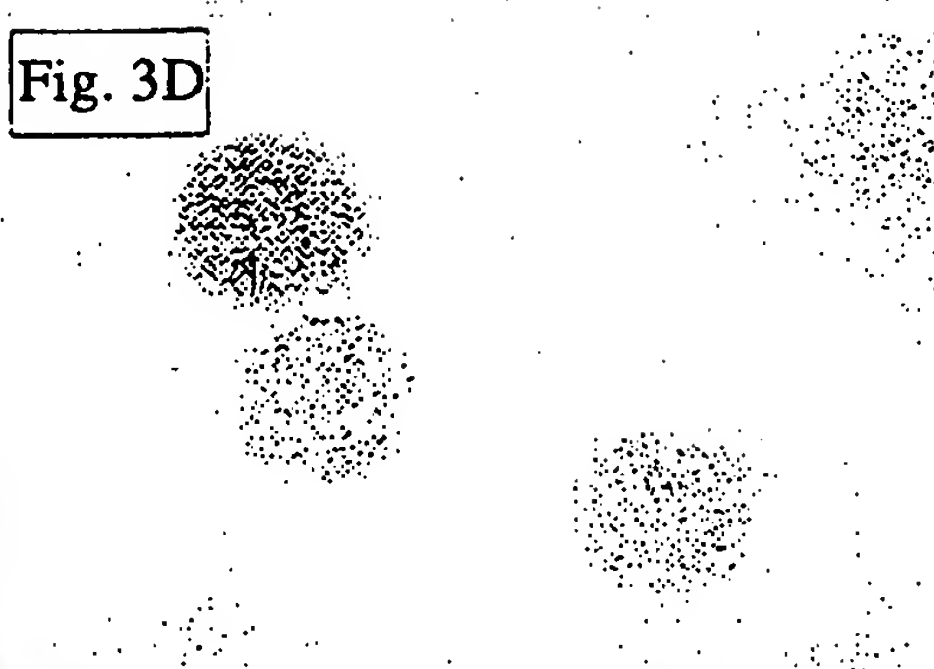


Fig.3B

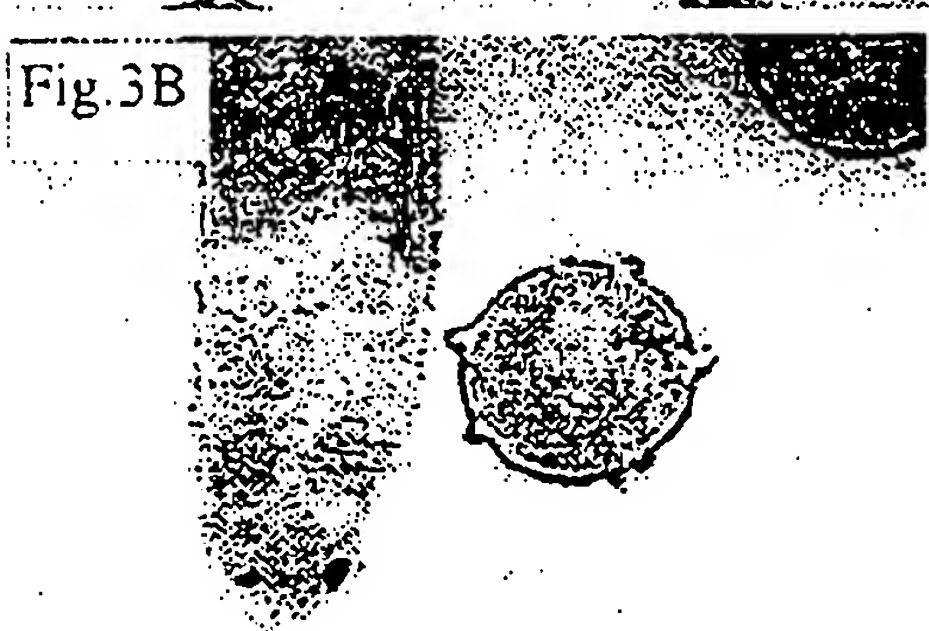


Fig. 3E

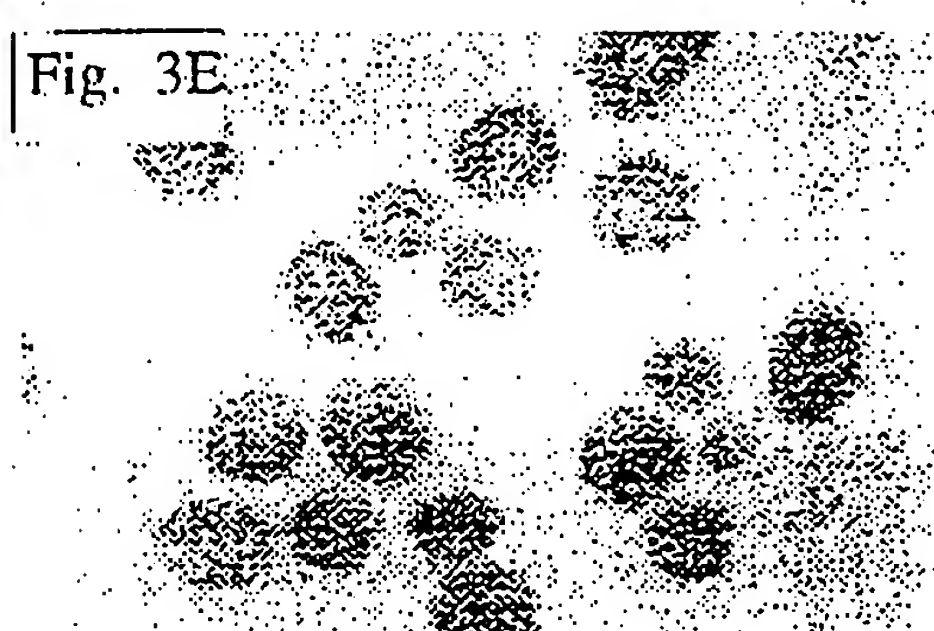
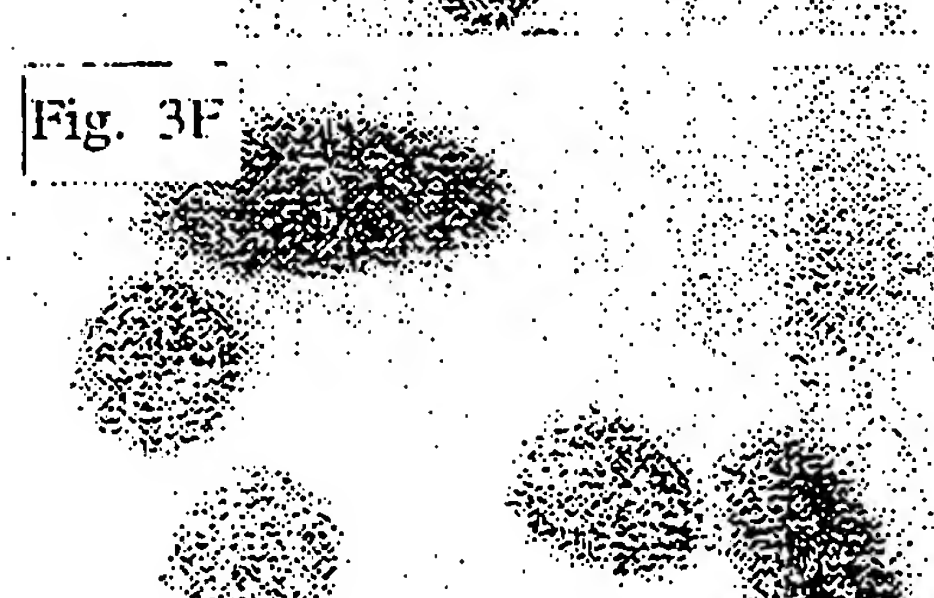


Fig. 3C



Fig. 3F



3/10

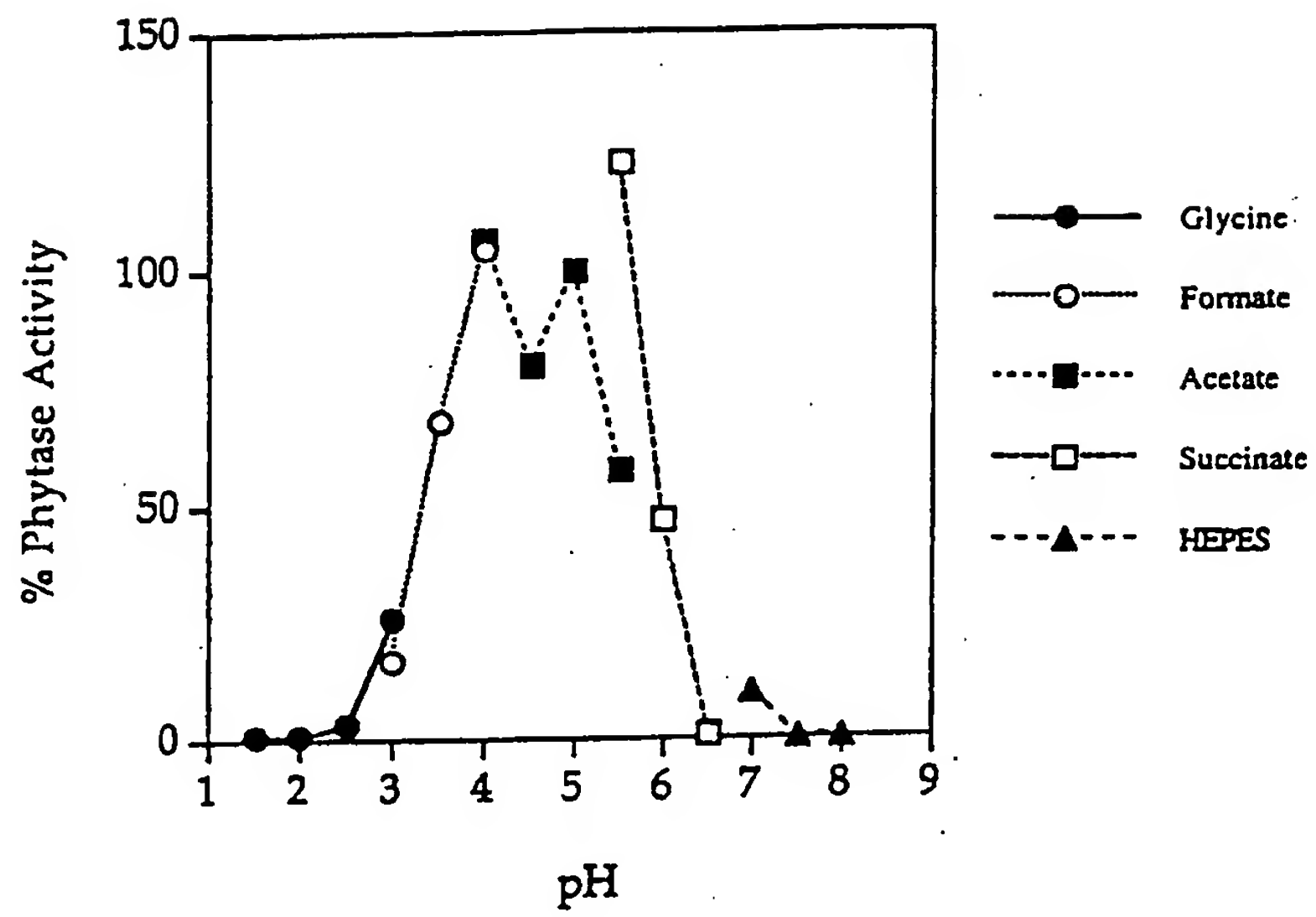


Figure 4

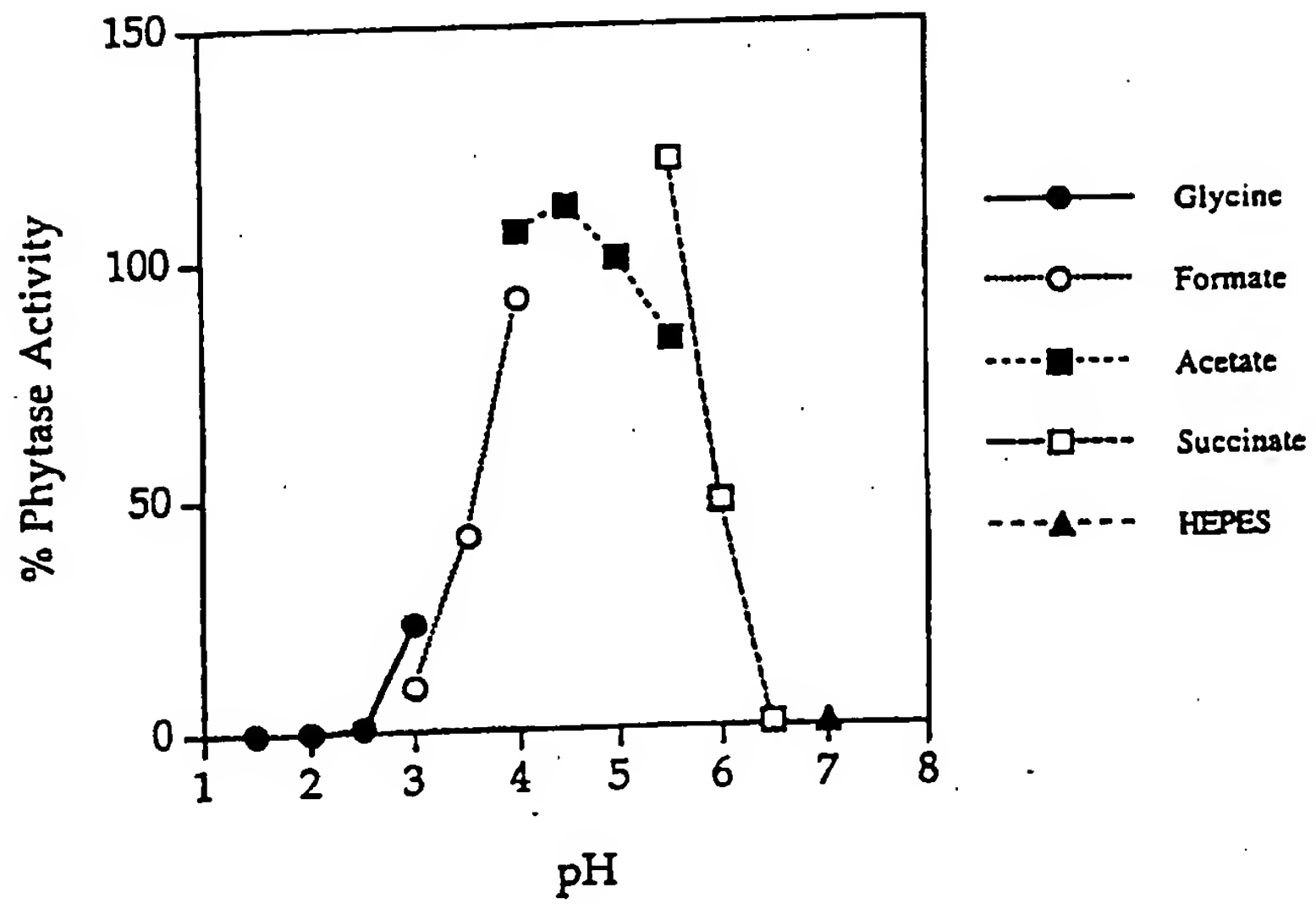


Figure 5

4/10

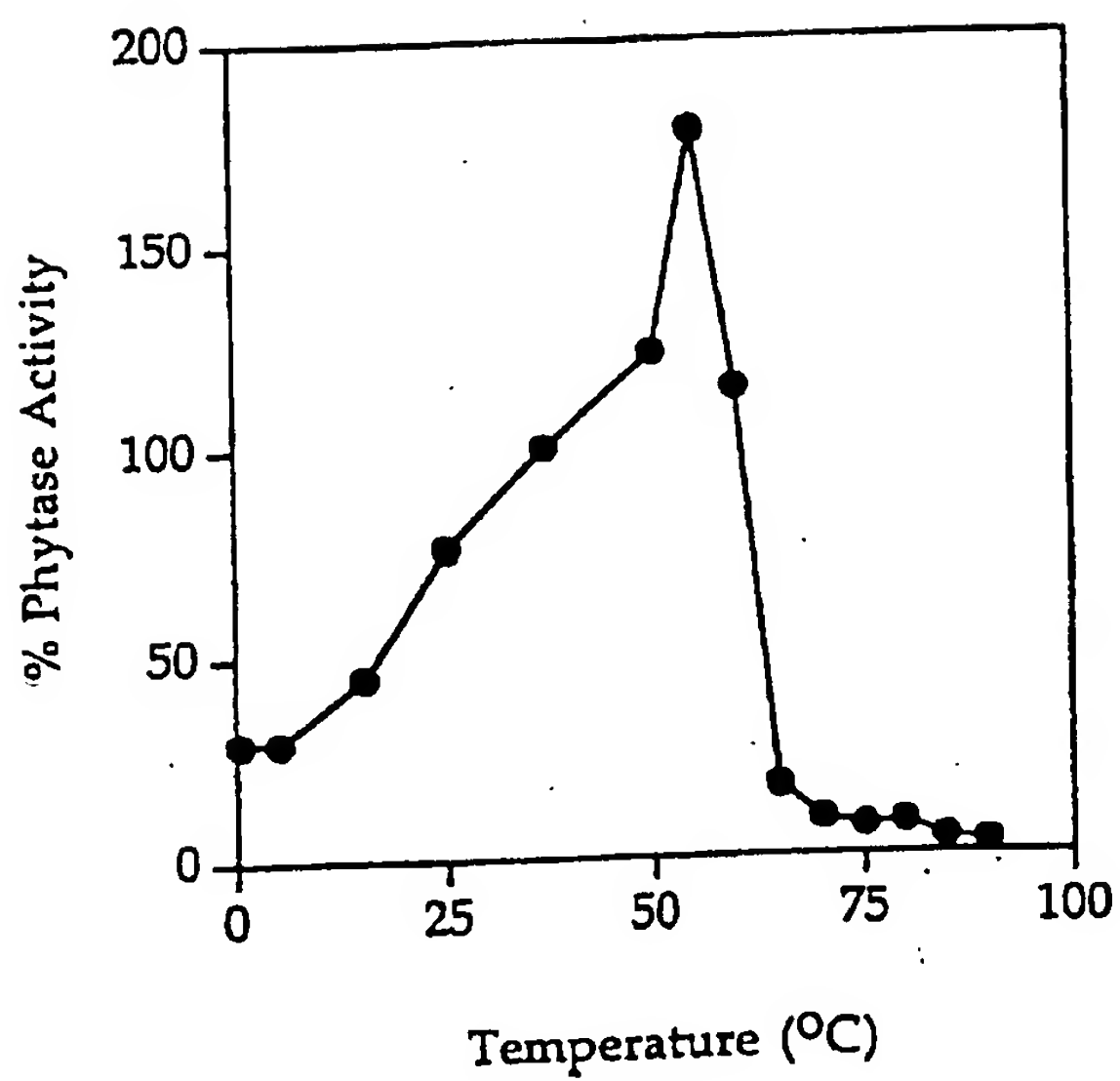


Figure 6

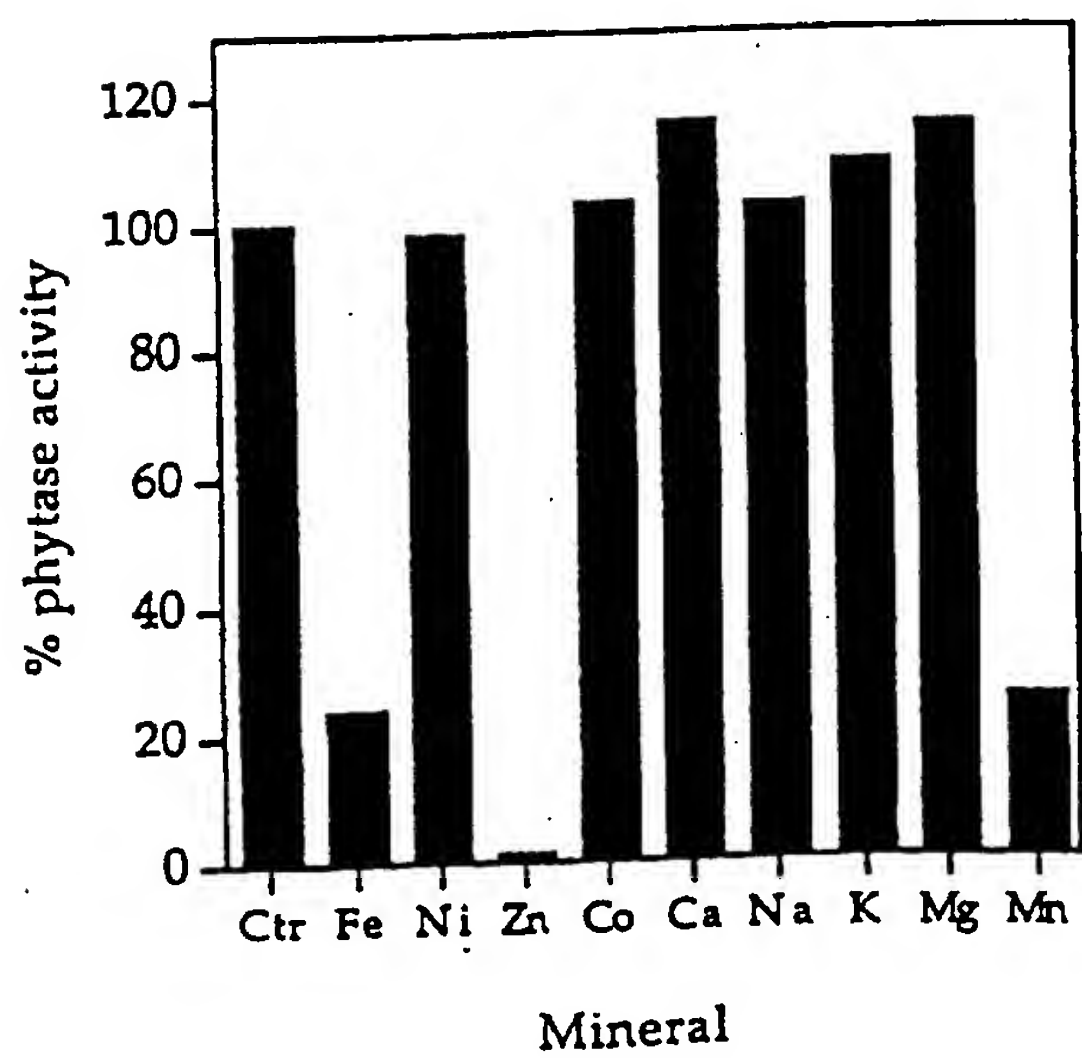


Figure 7

5/10

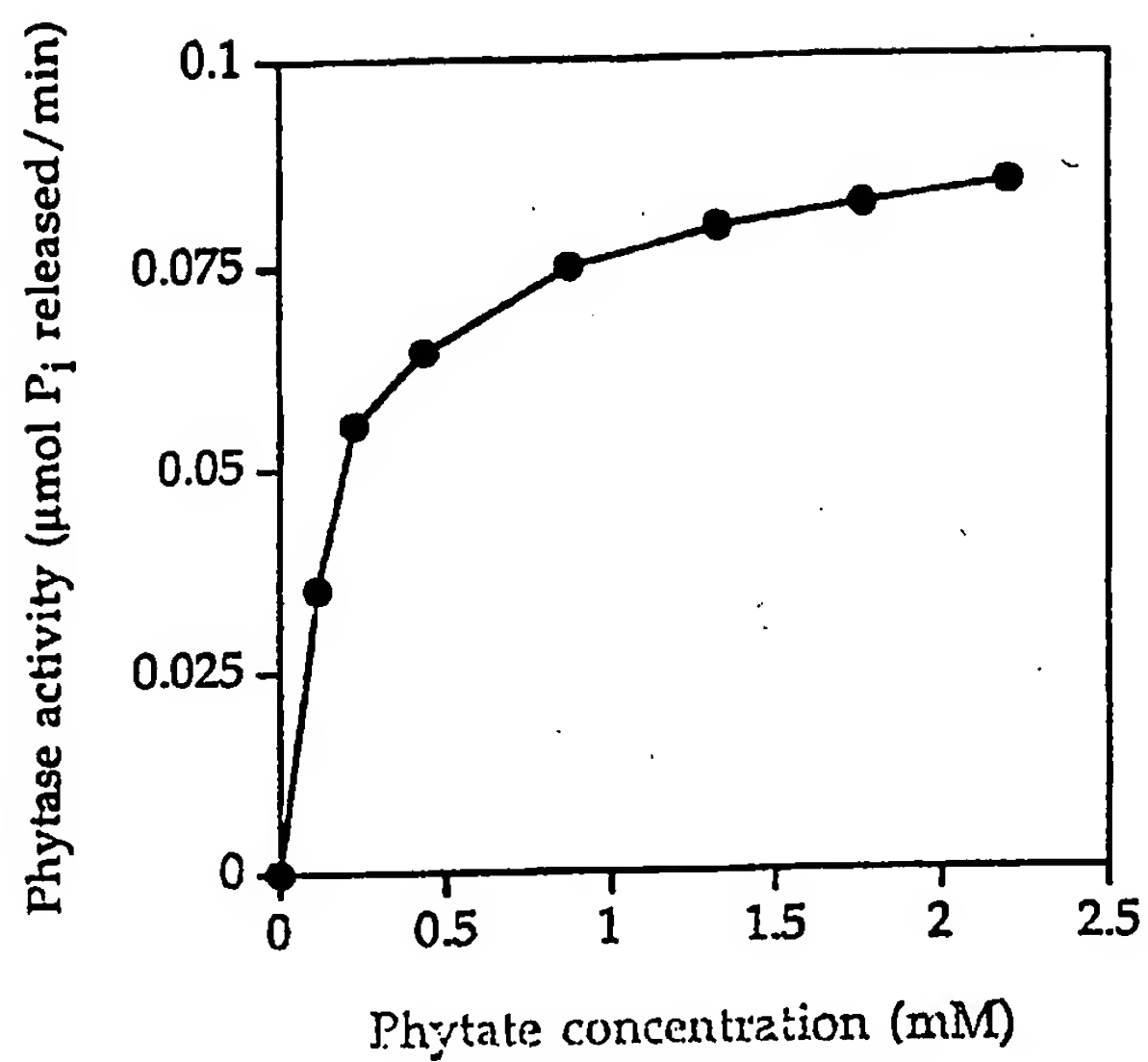


Figure 8

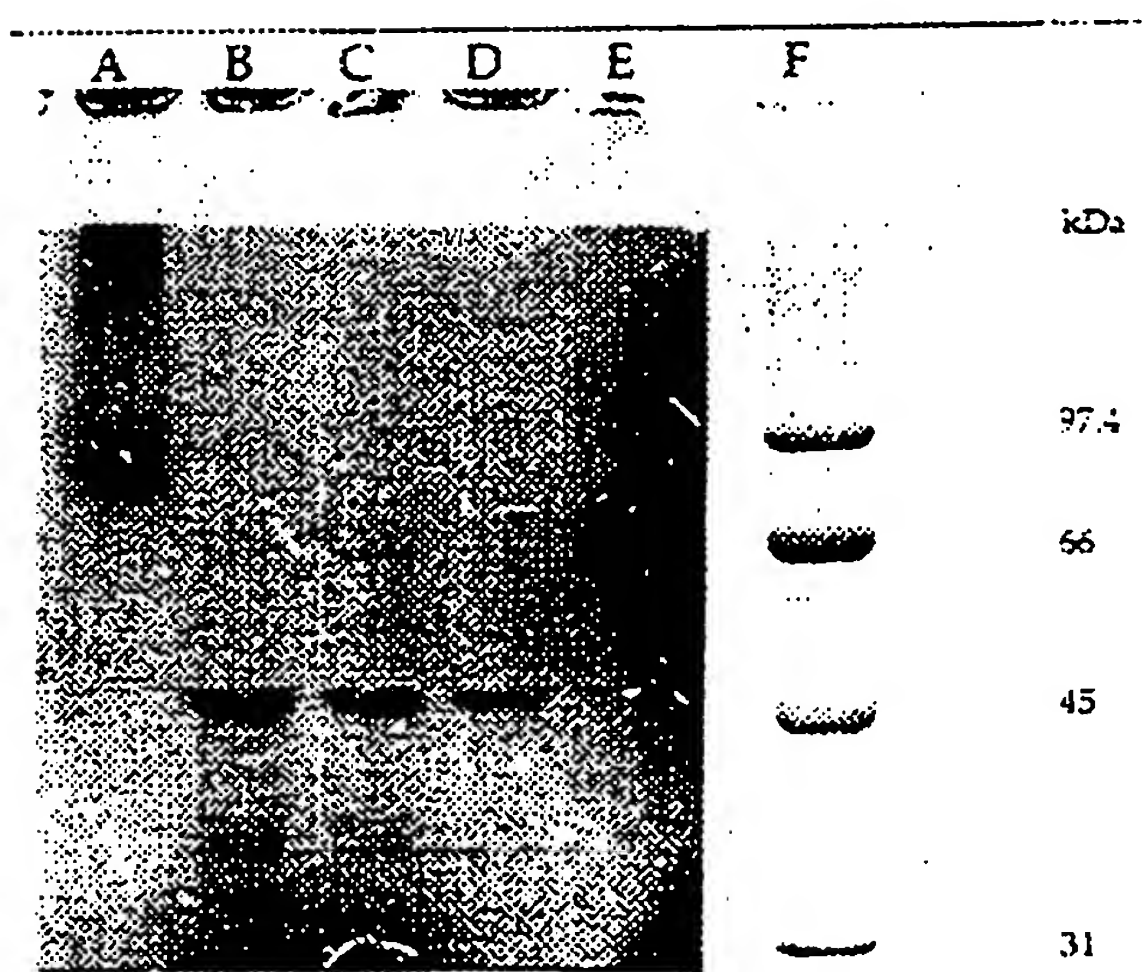


Figure 9

6/10

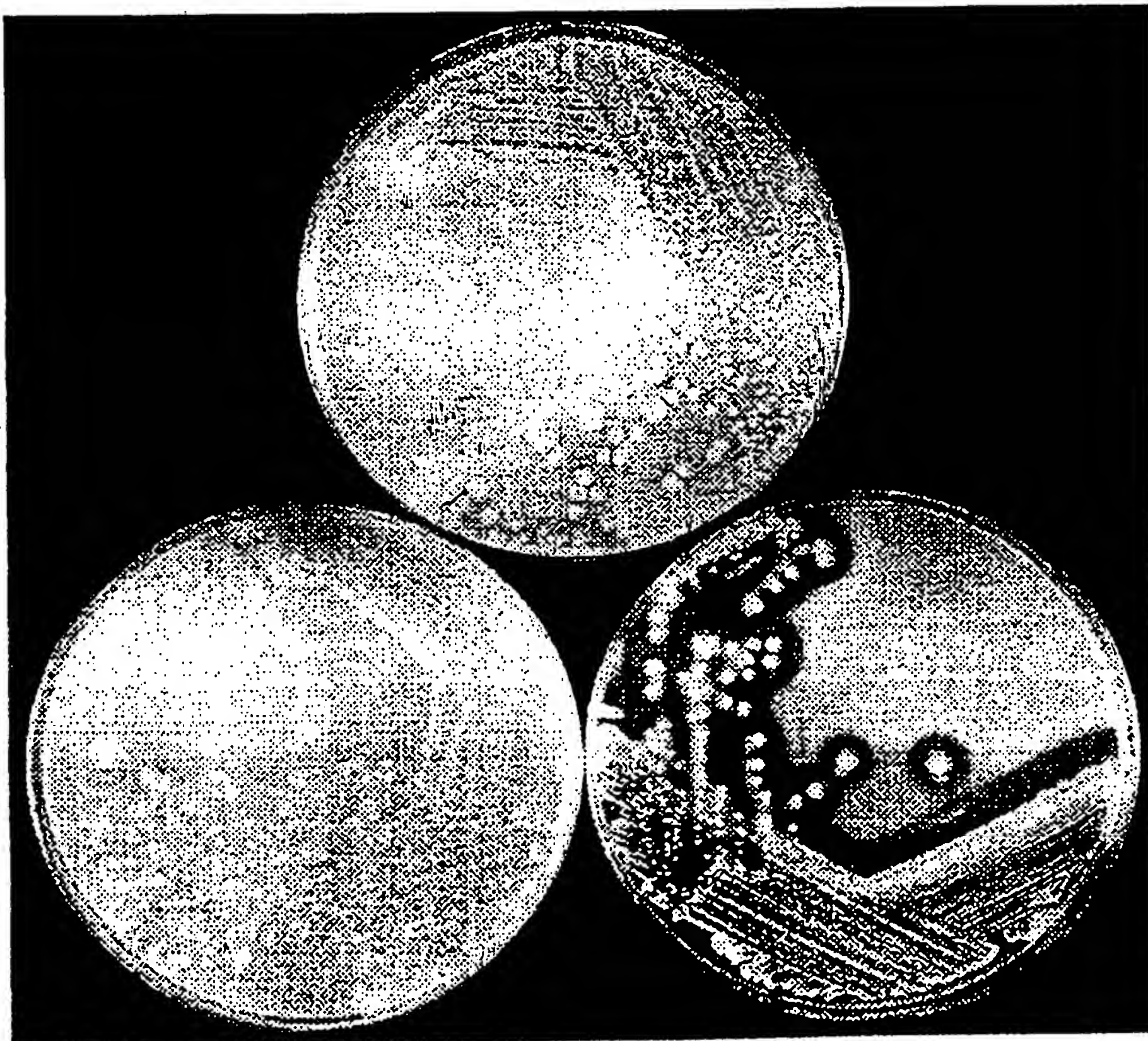


Figure 10

7/10

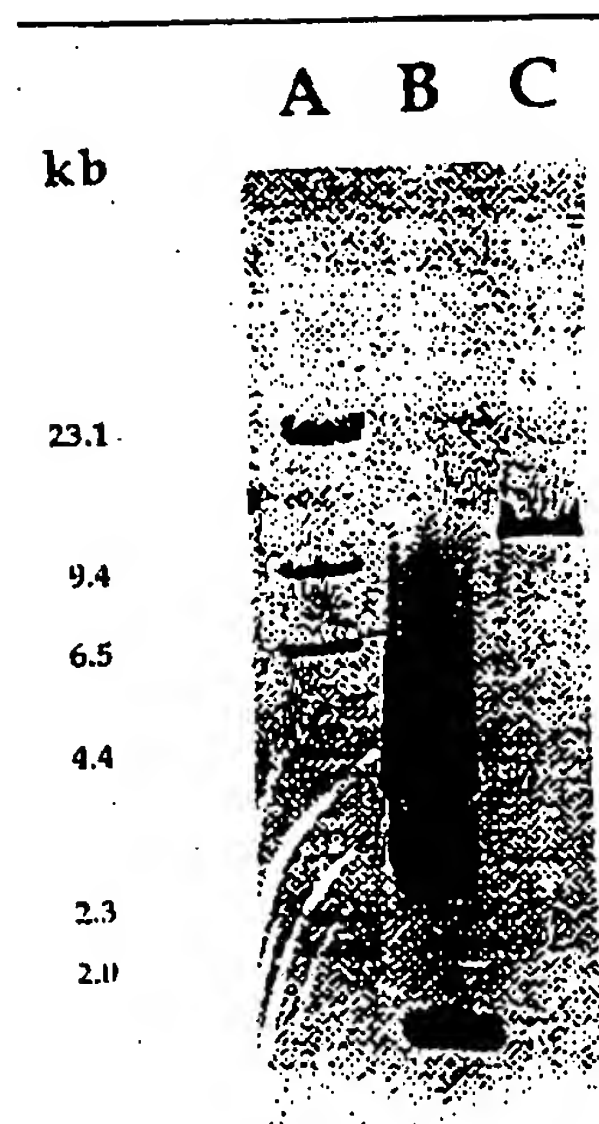


Figure 11

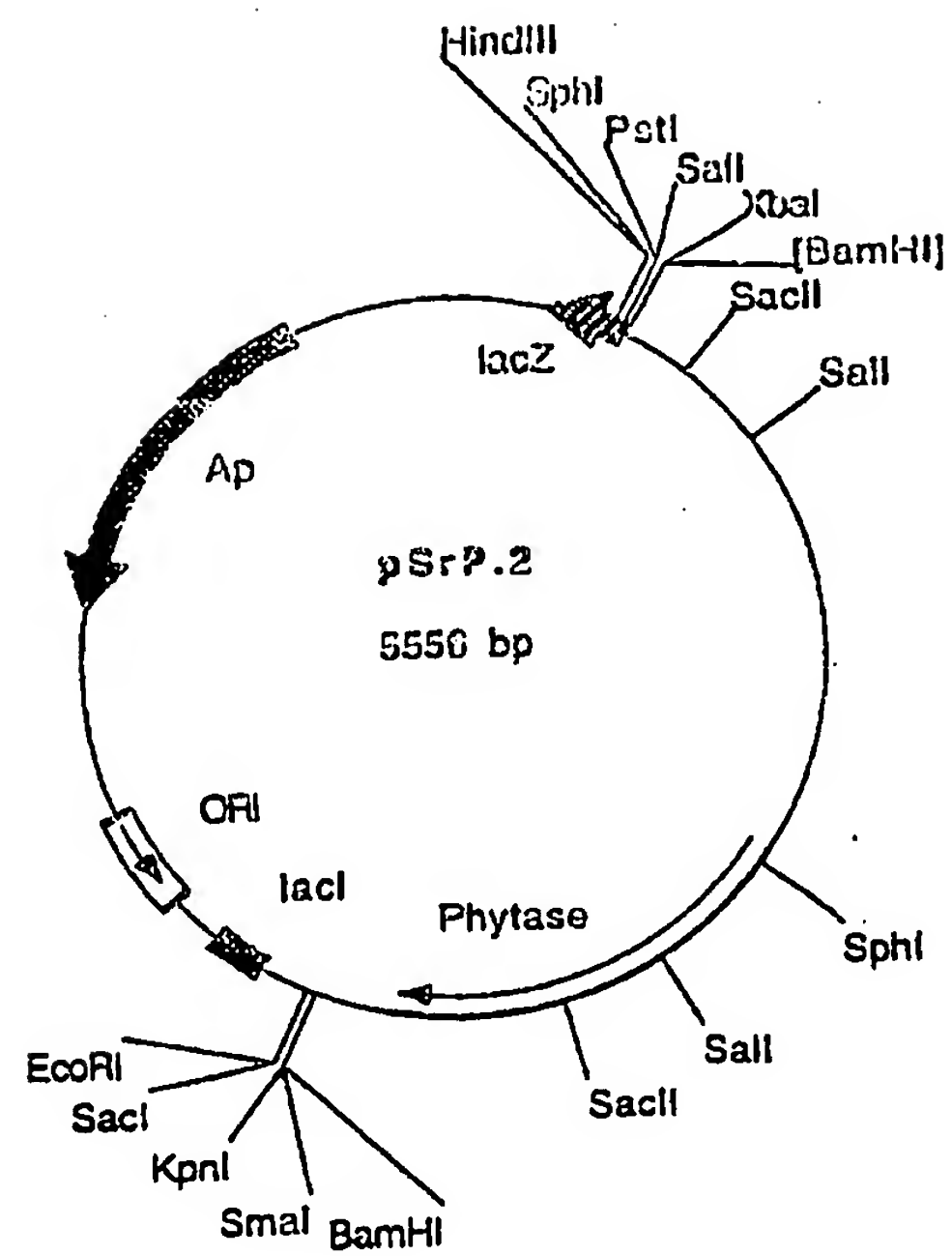


Figure 12

8/10

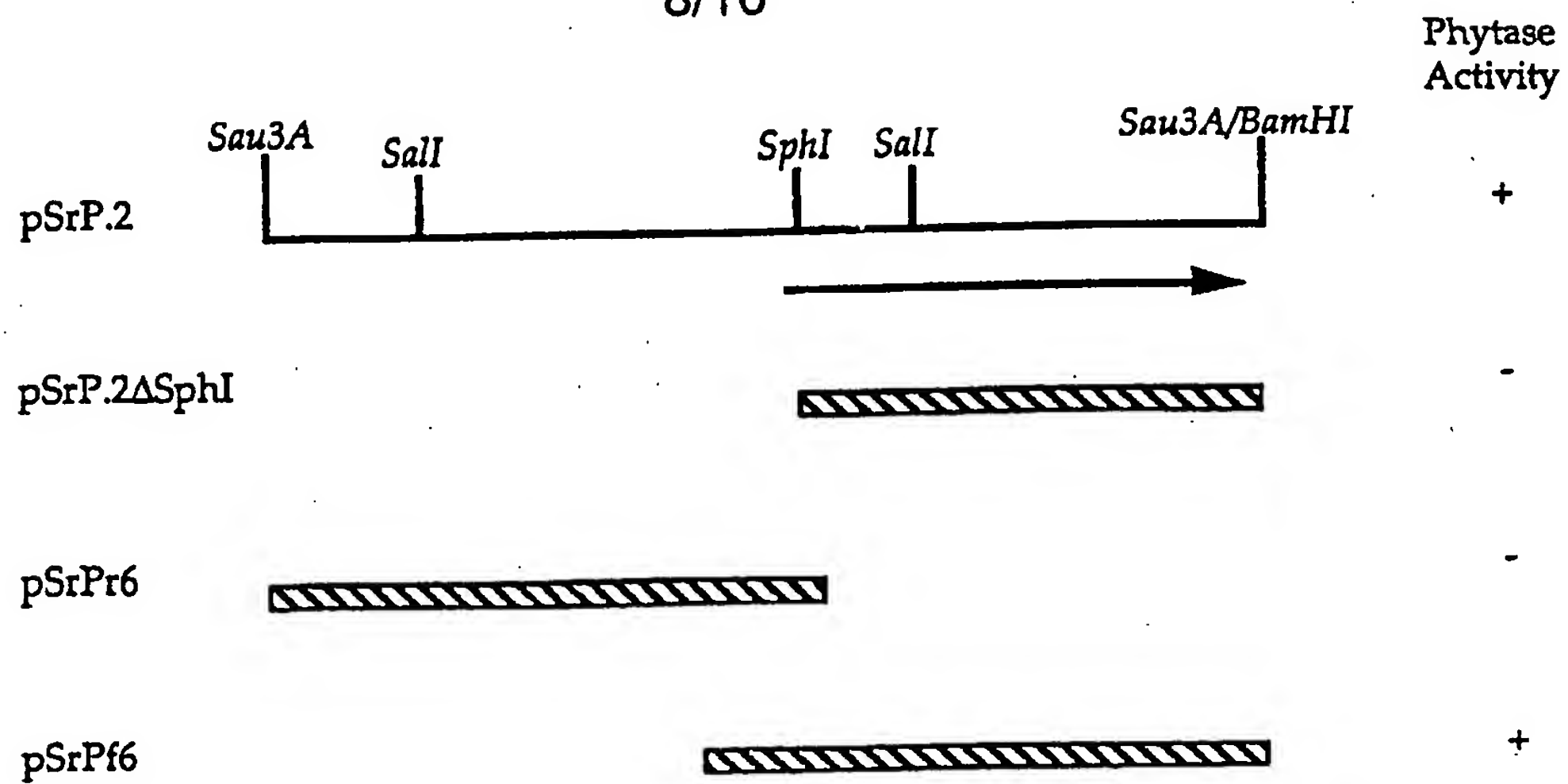


Figure 13

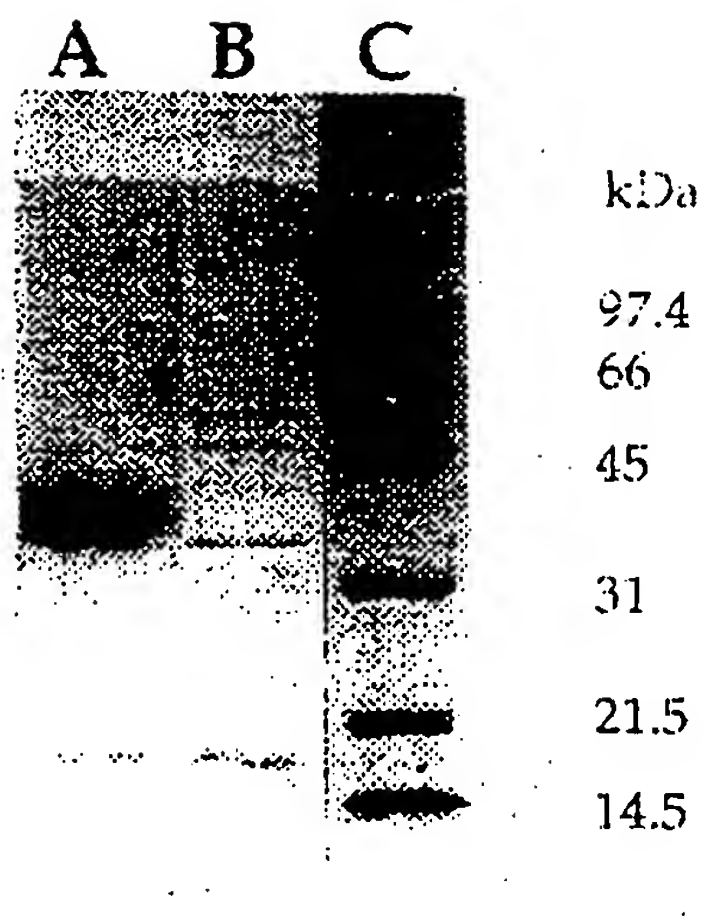


Figure 14

9/10

Figure 15

1 CGTCCACGGA GTCACCCTAC TATACGACGT ATGTGAAGTT CACGTCGAAG TTCTAGGGAA 60
 61 TCACCGATTG GTGCAGGATT TTACCACTTC CTGTTGAAGC GGATGAGAAG GGGAAACCGCG 120
 121 AAGCGGTGGA AGAGGTGCTG CACGACGGAC GATCGCGCTG AATGAATCAG TGCTTCCTAA 180
 R.B.S.
 181 CTATTGGGAT TCCGCGCAGA CGCGCGGATG GAGTAAAGGA GTAAGTTGTT ATG AAA TAC 239
 M K Y 3
 240 TGG CAG AAG CAT GCC GTT CTT TGT AGT CTC TTG GTC GGC GCA TCC CTC TGG 290
 4 W Q K H A V L C S L L V G A S L W 20
 291 ATA CTG CCG CAG GCC GAT GCG GCC AAG GCG CCG GAG CAG ACG GTG ACG GAG 341
 21 I L P Q A D A A K A P E O T V T E 37
 ↑
 342 CCC GTT GGG AGC TAC GCG CGC GCG GAG CGG CCG CAG GAC TTC GAG GGC TTT 392
 38 P V G S Y A R A E R P Q D F E G F 54
 393 GTC TGG CGC CTC GAC AAC GAC GGC AAG GAG GCG TTG CCG CGT AAT TTC CGC 443
 55 V W R L D N D G K E A L P R N F R 71
 444 ACG TCG GCT GAC GCG CTG CGC GCG CCG GAG AAG AAA TTC CAT CTC GAC GCC 494
 72 T S A D A L R A P E K K F H L D A 88
 495 GCG TAT GTA CCG TCG CGC GAG GGC ATG GAT GCA CTC CAT ATC TCG GGC AGT 545
 89 A Y V P S R E G M D A L H I S G S 105
 546 TCC GCA TTC ACG CCG GCG CAG CTC AAG AAC GTT GCC GCG AAG CTG CCG GAG 596
 106 S A F T P A Q L K N V A A K L R E 122
 597 AAG ACG GCT GGC CCC ATC TAC GAT GTC GAC CTA CGG CAG GAG TCG CAC GGC 647
 123 K T A G P I Y D V D L R Q E S H G 139
 648 TAT CTC GAC GGT ATC CCC GTG AGC TGG TAC GGC GAG CGC GAC TGG GCA AAT 698
 140 Y L D G I P V S W Y G E R D W A N 156
 699 CTC GGC AAG AGC CAG CAT GAG GCG CTC GCC GAC GAG CGG CAC CGC TTG CAC 749
 157 L G K S Q H E A L A D E R H R L H 173
 750 GCA GCG CTC CAT AAG ACG GTC TAC ATC GCG CCG CTC GGC AAG CAC AAG CTC 800
 174 A A L H K T V Y I A P L G K H K L 190
 801 CCC GAG GGC GGC GAA GTC CGC CGC GTA CAG AAG GTG CAG ACG GAA CAG GAA 851
 191 P E G G E V R R V Q K V Q T E Q E 207
 852 GTC GCC GAG GCC GCG GGG ATG CGC TAT TTC CGC ATC GCG GCG ACG GAT CAT 902
 208 V A E A A G M R Y F R I A A T D H 224
 903 GTC TGG CCA ACG CCG GAG AAC ATC GAC CGC TTC CTC GCG TTT TAC CGC ACG 953
 225 V W P T P E N I D R F L A F Y R T 241
 954 CTG CCG CAG GAT GCG TGG CTC CAT TTC CAT TGT GAA GCC GGT GTC GGC CGC 1004
 242 L P Q D A W L H F H C E A G V G R 258
 1005 ACG ACG GCG TTC ATG GTC ATG ACG GAT ATG CTG AAG AAC CCG TCC GTA TCG 1055
 259 T T A F M V M T D M L K N P S V S 275
 1056 CTC AAG GAC ATC CTC TAT CGC CAG CAC GAG ATC GGC GGC TTT TAC TAC GGC 1106
 276 L K D I L Y R Q H E I G G F Y Y G 292

10/10

1107 GAG TTC CCC ATC AAG ACG AAG GAT AAA GAT AGC TGG AAG ACG AAA TAT TAT 1157
293 E F P I K T K D K D S W K T K Y Y 309

1158 ACG GAA AAG ATC GTG ATG ATC GAG CAG TTC TAC CGC TAT GTG CAG GAG AAC 1208
310 R E K I V M I E Q F Y R Y V Q E N 326

1209 CGC GCG GAT GGC TAC CAG ACG CCG TGG TCG GTC TGG CTC AAG AGC CAT CCG 1259
327 R A D G Y Q T P W S V W L K S H P 343

1260 GCG AAG GCG TAA AAGCGCAGGC GCGCGCTCGG AGTCAGGGAA ATGGCGCTGC 1311
344 A K A * 346

1312 CAGCACGGGA CGCGCGGCGG CGGATGCTGC GCGGTCAGG GATGATTGAC GACAGCCAGA 1371

1372 GAAGAAAGGA TGGTTTTATG AGGTGGATCC 1401